UNIVERSITY OF TASMANIA

THE PATHOGENESIS OF EPIZOOTIC ULCERATIVE SYNDROME (EUS) WITH PARTICULAR REFERENCE TO FACTORS POTENTIALLY INFLUENCING OUTBREAKS OF THE DISEASE IN THE PHILIPPINES

by

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Submitted in fulfilment of the requirements
for the Degree of Doctor of Philosophy

University of Tasmania
January 2001

DECLARATION

I hereby declare that this Thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due reference is made in the text of the Thesis.

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ABSTRACT

Epizootic ulcerative syndrome (EUS) outbreaks in the Philippines had been consistently associated with periods of decreasing and fluctuating water temperature usually after the rainy season. Therefore, one of the main objectives of this study was to examine the influence of low and highly fluctuating temperature on the pathogenesis of the disease based on the histopathological and epidermal changes. Also, the influence of water temperature variations on the nonspecific immune responses of a fish exposed to *Aphanomyces invadans* was determined. Moreover, the efficacy of four therapeutic substances to enhance the defense mechanisms of susceptible fish against EUS was assessed.

A model of the sequential pathology of EUS was developed and described in three spot gourami *Trichogaster trichopterus* and sand whiting *Sillago ciliata.* Both species of fish exhibited histopathological changes typical of EUS, such as chronic granulomatous response and inflammatory cell infiltration in the muscle and skin tissues, at day 8 post-inoculation of 55 to 70 spores/fish. From the results of comparative granuloma counts and percentage of cellular infiltration in a sampled lesion area using image analysis, it was shown that the three-spot gouramis developed a more vigorous response than the sand whiting.

Based on the EUS model, the effects of either rapid or gradual decrease in temperature on the histopathology of EUS in sand whiting was documented. Qualitative and quantitative histopathological examinations revealed a marked delay in the inflammatory response of fish maintained at low temperature. EUS lesions were also induced in skin-abraded three-spot gouramis exposed to motile *A. invadans* zoospores, with higher mortality in fish subjected to daily temperature variations (26 °C to 19 °C) than in fish held at 26 °C. Histopathological examination also revealed minimal inflammatory cell infiltration and delayed mycotic granuloma formation with extensive tissue damage in temperature-stressed fish.

Fish oil, L-cysteine ethyl ester, yeast glucans and levamisole were trialled as prophylactics against EUS. Only yeast glucans and levamisole gave encouraging results.

Results from this study showed that low and fluctuating temperature could affect the host's defense mechanisms against EUS and that preventive measures should be aimed at the maintenance of the epidermal integrity and improvement of the nonspecific immune response of fish.

ACKNOWLEDGMENTS

I wish to thank the following for their valuable support and assistance during the course of this research:

Dr. Barry L. Munday, my supervisor, for his unwavering support, guidance and encouragement, and for his advice and critical review of the draft manuscript;

Drs. Barbara Nowak and Natalie Moltschaniwsky for their suggestions on the statistical analyses of quantitative data;

Dr. R. Callinan, of the NSW Fisheries, for providing background information on EUS and for the *Aphanomyces invadans* isolate (24P) which was originally isolated by Ms. Annette Thomas of the DPI Queensland; Dr. Jim Lilley for providing a replacement culture of isolate 24P from Stirling University; the sand whiting fry were from the Bribie Island Aquaculture Research Centre (DPI Queensland); Aquagard was from the Aquatic Diagnostics Services.

Technical assistance was provided by Mr. Rhys Hauler with the preparation of the low fish oil diet; Mr. Greg Kent with the image analyser; Mr. Craig Thomas with the rearing of the sand whiting fry; and Mr. Rob Gurney, former technician of Fish Health Laboratory, with histological techniques;

Vera, Maureen, Reiny, Jenny Cleary, Ruth Mollison, Jo, Tiina, Andy, Jacqui, Phil, Niall, Dina, Mehd and Felipe and other colleagues at the School of Aquaculture for the good company and fun they provided throughout the years;

Ms. Annabel Tyson, for managing the research funds;

The Australian Centre for International Agricultural Research for the research funds and financial assistance for conference participation;

and lastly, to my family, for their love, encouragement and prayers.

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AB (+)	-alcian blue (+) mucous cells or acidic mucopolysaccharide
AB/PAS	-alcian blue/periodic acid-Schiff's stain
ACIAR	-Australian Centre for International Agriculture Research
ACP	-alternative complement activity
ADB	-Asian Development Bank
ANOVA	-analysis of variance
APW	-autoclaved pond water
BAPNA	-Nα-benzoyl-dl-arginine p-nitroanilide
BSA	-bovine serum albumin
CCP	-classical complement pathway
DHA	-docosahexanoic acid
DMSO	-dimethyl sulfoxide
DO	-dissolved oxygen
DPI	-Department of Primary Industries
EDTA	-ethylene diamine tetraacetic acid
EGTA	-ethylene glycol-bis(β-aminoethyl ether) N,N,N^{1},N^{1}-tetraacetic acid
EPA	-eicosapentanoic acid
EUS	-epizootic ulcerative syndrome
FAO	-Food and Agricultural Organisation
FPS	-fish physiological saline
FT	-fluctuating temperature
GYA	-glucose-yeast agar
HBSS	-Hank's balances salt solution
L-CEE	-L-cysteine ethyl ester
MAF	-macrophage activating factor
MG	-mycotic granulomatosis
NACA	-Network of Aquaculture Centres in Asia-Pacific
NH_3-N	-ammonia-nitrogen
NO_2^-	-nitrite
ODA	-Overseas Development Administration of the United Kingdom
PAS	-periodic acid-Schiff's stain

PAS(+)	-mucous cells with neutral polysaccharides
PBS	-phosphate-buffered saline
p.i.	-post-injection or post-inoculation
PMA	-phorbol-12-myristate 13-acetate
PMN	-polymorphonuclear neutrophil
RAPD-PCR	-random amplification of polymorphic DNA-polymerase chain reaction
RaRBC	-rabbit red blood cells/erythrocytes
RBC	-red blood cells/erythrocytes
ROS	-reactive oxygen species
RSD	-red spot disease
SC	-sacciform cells
SDS-PAGE	-sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	-superoxide dismutase
TMC	-total mucous cell count
UM	-ulcerative mycosis
WBC	-white blood cells/leukocytes

Chapter One

General Introduction

1.1 Background on Epizootic Ulcerative Syndrome (EUS)

Various cutaneous ulcerative diseases of fish which consistently involve an oomycete fungal element, have been reported since the early 1970's in Japan as mycotic granulomatosis or MG (Miyazaki and Egusa 1972, cited by Miyazaki 1994), in 1972 in Australia as red spot disease or RSD (McKenzie and Hall 1976), in 1984 in the southeastern coast of the United States as ulcerative mycosis or UM (Noga and Dykstra 1986) and early 1980's and onwards in southeast and western parts of Asia as epizootic ulcerative syndrome or EUS (Lilley *et al.* 1992; Bondad-Reantaso *et al.* 1992; Roberts *et al.* 1993). Pathological and epizootiological evidences indicate that these ulcerative conditions are indistinguishable and may in fact be just one syndrome (ODA 1994). A wide range of freshwater and estuarine fish species is susceptible to EUS and at least 100 such species have been identified in Asia and Australia (Lilley *et al.* 1998).

To put the economic importance of EUS in context, it should be remembered that the world fisheries production in 1996 reached 121 million tons, 20% of which was contributed by aquaculture (FAO 1999). In Asia alone, aquaculture provided 25.0 million tonnes in 1995, valued at US $34.8 thousand million (Tacon 1997). However, it has been reported that an estimated annual loss of US $3 thousand million in aquaculture production is incurred mainly due to fish diseases and environment-associated problems in Asian countries (ADB/NACA 1996; cited by Subasinghe *et al.* 1997). In the same report, epizootic ulcerative syndrome (EUS) was identified as one of the most serious finfish diseases in the Asia-Pacific region. Due to the presence of large skin ulcers, the affected fish were unsaleable and mortality rates during outbreaks were often high, causing tremendous socio-economic problems in the region. It has spread to different Asia-Pacific countries over the last two decades, with Pakistan being the last reported country to be affected by EUS outbreaks (Lilley *et al.* 1998).

The devastating effects of the annual EUS outbreaks in Asia has caused much concern, especially in a region where aquaculture constitutes a substantial proportion of the economic sector and where most of the population depends on fish as the main source of protein. Initially, the Consultation of Experts on

Ulcerative Fish Diseases in 1986, proposed that a rhabdovirus was the primary causative agent (Frerichs *et al.* 1986) and that there is an association between EUS outbreaks and environmental factors (FAO 1986). But it was recommended that more intensive studies be undertaken concerning the aetiological and environmental factors, which may be related with EUS outbreaks. It was also suggested that possible control and preventive measures against the syndrome be identified.

1.1.1 Aetiology

Different infectious agents have been reported to be associated with EUS. However, the most common feature of the syndrome is the presence of fungal hyphae in the lesions. The earliest report on the involvement of a pathogenic fungus with an ulcerative disease was from Japan (Miyazaki and Egusa 1972) affecting both wild and farmed freshwater species (Miyazaki and Egusa 1973a; Miyazaki and Egusa 1973b; Miyazaki and Egusa 1973c; Hatai *et al.* 1977). Hatai *et al.* (1984) identified the fungus as *Aphanomyces* and proposed the name *Aphanomyces piscicida* in 1980. Mckenzie and Hall (1976) also indicated the presence of a fungus with the same morphology as *Aphanomyces* in ulcerated sea mullet in Queensland. Reports from other areas in Australia, including Tasmania (Munday 1985) and the Northern Territory (Pearce 1990), also mentioned fungal involvement in fish skin ulcers. But it was Fraser *et al.* (1992) who identified the fungal isolates from different estuarine fish with red spot disease as *Aphanomyces*. Likewise, Noga and Dykstra (1986) reported the consistent presence of oomycete fungi in Atlantic menhaden with UM. In 1991, *Aphanomyces* sp. were recovered from EUS-affected fish collected from freshwater and brackishwater environments in the Philippines and were shown to be very closely related to the Australian *Aphanomyces* sp isolates, as shown by the peptide bands profile using SDS-PAGE (Callinan 1994b; Callinan *et al.* 1995a). Roberts *et al.* (1993) and Willoughby *et al.* (1995) also reported the isolation of this fungus from freshwater fish in Bangladesh and Thailand. Although three species of fungi, namely *Achlya, Saprolegnia* and *Aphanomyces* have been isolated from infected fish in Asia (Roberts 1994; Vishwanath *et al.*

1998), it is the latter which was identified to be the invasive agent causing the granulomatous reactions in the dermis and superficial muscles of susceptible fish.

Aphanomyces belongs to Class Oomycete, specifically under the group of aquatic fungi, Saprolegniales (Webster 1980). Willoughby *et al.* (1995) described the pathogenic *Aphanomyces* sp. associated with EUS as having broad, aseptate, isodiametric hyphae 11.7-16.7 μm across when examined in fish tissues, but narrower hyphae, 8.3 μm across, when observed in cultures and the name *Aphanomyces invaderis* was proposed for this species. Usually a single row of zoospores is discharged through evacuation tubes of the narrow and complex zoosporangium at the tip of the hyphae (Neish and Hughes 1980; Fraser *et al.* 1992; Willoughby *et al.* 1995). However, *Aphanomyces* isolates from Thailand and Bangladesh have 4 evacuation tubes while the Australian and Philippines isolates usually have 1-2 evacuation tubes (Callinan *et al.* 1995a). The primary zoospores usually encyst and form clusters or balls at the evacuation tubes. The cysts, 6.7-10 μm in diameter, subsequently release secondary, motile zoospores with two flagella (Willoughby *et al.* 1995). Although hooked hairs, which could aid in attachment, were observed in Saprolegniales species (Webster 1980; Willoughby 1994), Noga (1993) suggested that these might not be important in their pathogenicity.

It appears that growth of the pathogenic *Aphanomyces* is dependent on temperature. Fraser *et al.* (1992) reported that the three fungal isolates from Australia grew well between 13-31 °C, but not at 3 or 37 °C. Moreover, Roberts *et al.* (1993) conducted temperature-growth investigations among saprophytic and pathogenic *Aphanomyces*. The pathogenic species was slow growing between 10 to 20 °C and died at 37 °C, while saprophytic species grew well at 37°C (Willoughby 1994). Sporulation was also reported to be affected by temperature. At 10 °C, the pathogenic species could dehisce and release motile zoospores, but not at 37 °C. In contrast, the saprophytic isolate could open and release motile zoospores at 37 °C but not at 10 °C. At 25-31 °C, both species dehisced well (Roberts *et al.* 1993). Vegetative growth is also optimal at 21-25 °C for *Aphanomyces* isolated from ulcerated Atlantic menhaden (Dykstra *et al.* 1986).

Fraser *et al.* (1992) also reported that growth of *Aphanomyces* was completely inhibited in medium with 12‰ NaCl, and sporulations occurred only in fresh water and in sporulating medium with 2‰ NaCl. Most members of the family Saprolegniaceae, which include *Aphanomyces*, are incapable of surviving or sporulating in high saline environments (Pearce 1990). However, Hearth and Padgett (1990) noted that an *Aphanomyces* isolate from UM-affected fish could survive and germinate in full strength seawater provided that proper nutrients are present in the environment. This is consistent with an earlier study by Dykstra *et al.* (1986) who indicated that low levels of NaCl (2-4‰) could stimulate growth and sporulation of this fungus. Shafer *et al.* (1990) suggested that this enhanced salinity tolerance may be due to some specific proteins being synthesized by the UM-associated fungus during salt stress and cannot be detected in fungi recovered from freshwater environment. Recently, *Aphanomyces* spp. were isolated from ulcerated striped mullet, *Mugil cephalus*, and thin lip grey mullet, *Liza ramada*, in Egypt during the 1997 winter season (Shaheen *et al.* 1999). The isolates were reported to grow at temperatures up to 30 °C and at 2-8 ‰ salinity.

Recent findings comparing the characteristics of the pathogenic *Aphanomyces* strains obtained from EUS-, RSD-, MG- and UM-affected fish have been reported. Lilley and Roberts (1997) carried out laboratory trials to compare the pathogenicity of different *Aphanomyces* isolates obtained from EUS-, RSD-, MG- and UM-affected fish, saprophytic *Aphanomyces* and other saprolegniaceous species. Results showed that only the *Aphanomyces* isolates from EUS-, RSD- and MG-affected fish invaded snakehead muscle and induced the development of typical EUS lesions. Moreover, growth on different culture media and temperatures further confirmed the similarities of the *Aphanomyces* species involved in EUS, RSD and MG lesions. Lilley and Inglis (1997) also showed that the UM-related *Aphanomyces* differed from the other pathogenic *Aphanomyces* species in terms of salt tolerance, with the EUS, RSD and MG strains being more susceptible to salt treatment. Furthermore, analysis by SDS-PAGE, lectins/receptor profiles and reaction with polyclonal antisera against the various fungi revealed the same protein and carbohydrate profile for the *Aphanomyces* isolates from EUS-, RSD- and MG-affected fish (Lilley *et al.* 1997b). It was also shown by using RAPD-PCR that fungal isolates from Japan, Australia and some

Asian countries were very likely to be "clonets" as they did not exhibit genetic variations (Lilley *et al.* 1997a). Since neither *Aphanomyces piscicida* nor *Aphanomyces invaderis* were valid under the International Code for Botanical Nomenclature, the name *Aphanomyces invadans* was proposed to designate the EUS, RSD and MG fungal pathogens.

Other infectious agents have been associated with EUS outbreaks. A number of viruses (birnavirus, rhabdovirus, retrovirus) have been isolated but the inconsistent and low recovery rate and unsuccessful initial experimental attempts to produce the disease using these viruses indicate that a viral agent seems unlikely to be the necessary EUS pathogen (Frerichs *et al.*1986; Frerichs *et al.* 1989; Lilley *et al.* 1992; Frerichs *et al.* 1993; Frerichs 1994; Millar 1994b). However, Kanchanakhan (1996) reported the recovery of rhabdoviruses from EUS-affected fish during the 1993-94 and 1995-96 epizootics in Thailand, and typical EUS lesions were induced through experimental exposure of snakehead fish to one of these rhabdoviruses and *A. invadans.* Lio-Po *et al.* (1999) also reported virus recovery in catfish in a cohabitation experiment with EUS-affected snakehead. However, even though lesions were induced, the typical EUS pathological sign, the presence of mycotic granulomas, was not demonstrated from the fish samples in that instance (Cruz-Lacierda and Shariff 1995). Kanchanakhan *et al.* (1999) were able to induce EUS lesions in snakehead fish by injecting a rhabdovirus intramuscularly, followed by exposure to *A. invadans* spores. The virus was able to induce skin damage, allowing the invasion of the fungal pathogen. Hence, this virus can be considered as one of predisposing factor prior to *A. invadans* infection, but not necessarily the main aetiological agent.

Opportunistic types of bacteria, predominantly *Aeromonas hydrophila* in freshwater and *Vibrio* spp. in estuarine environment, have also been associated with EUS (Rodgers and Burke 1981; Llobrera and Gacutan 1987; Boonyaratpalin 1989; Bondad-Reantaso *et al.*1992). However, pathogenicity studies failed to show a clear relationship between these bacteria and EUS and they were thus dismissed as secondary pathogens (Callinan and Keep 1989; Lilley *et al.* 1992; Millar 1994a, Callinan 1994a). Likewise, parasitological investigations failed to

establish the relationship of parasites with EUS outbreaks, but present evidence shows that parasite load can make fish more susceptible to EUS (Mckenzie and Hall 1976; Callinan and Keep 1989; Bondad-Reantaso 1991; Subasinghe 1993).

1.1.2 Environment and climatic factors

The relationship of EUS outbreaks and environmental factors has also been investigated. Studies undertaken in Australia showed that the occurrence of ulcerative epizootics in estuarine areas was related to major rainfall events and changes in water quality such as low and fluctuating temperatures, depressed salinity, low dissolved oxygen and pH (Rodgers and Burke 1981; Virgona 1992). In India, EUS outbreaks in estuarine fish were associated with monsoon rains and drop in salinity (Mohan and Shankar 1994). Callinan et al. (1995b) suggested that there was an association between EUS outbreaks in Australia and the Philippines and the presence of acid sulphate soils in river catchments. However, the pH ranged from 7.1-7.5 during an outbreak in a lagoon in Buguey, in northern Philippines, which suggests that low pH and acid runoff may not be a significant factor in EUS incidence in this country. Subsequent studies in northern New South Wales, Australia (Callinan et al., 1996; Callinan 1997) confirmed that after a rainfall event, acid-sulfate soil runoff in catchments, usually with pH 5.0- 6.3, could cause damage on the epidermis of fish serving as entry points for *A. invadans* zoopores. Apparently, *A. invadans* can produce motile zoopores at this pH range. Low dissolved oxygen was also implicated in the development of EUS lesions in sea mullet in estuarine areas (Callinan *et al.* 1989) but initial laboratory experiments involving manipulations of dissolved oxygen content of tank water and the addition of motile zoospores failed to induce EUS lesions (Callinan 1997). As these studies were inconclusive, more observations are required to clarify this aspect of EUS epidemiology.

Studies on environmental and climatic variables that might play a role in EUS outbreaks in Southeast and South Asia have also been undertaken. Based on the findings from the NACA Regional Research Programme on Relationships between EUS in Fish and the Environment, conducted in 11 Asian countries, Phillips (1994) reported decreasing alkalinity, water hardness and chloride

concentrations prior to EUS outbreaks. Moreover, in the Philippines and Bangladesh, EUS occurred during periods of low and highly fluctuating diurnal temperature (Bondad-Reantaso et *al.*1992; Phillips 1994). EUS outbreaks were also reported during periods of decreasing temperatures in Thailand, India and Sri Lanka (Balasuriya 1994; Chinabut 1994; Das 1994). In addition, outbreaks of the disease were seasonal in all countries, with EUS usually occurring during months after major rainfall events. In the same report, it was established that there was no correlation between EUS outbreaks and the use of fertilizers, pesticides and other agro-chemicals. Ahmed and Rab (1995), included pond management, soil characteristics and farming history as factors in EUS occurrences in Bangladesh. From 1993-1996, the Australian Centre for International Agricultural Research funded a collaborative project involving researchers from Australia, Indonesia and the Philippines. Based on the data obtained during the trials, no water quality parameter, except water temperature, seems to be associated with EUS outbreaks in ponds or rice-fish plots in Southeast Asia (Callinan undated).

In relation to UM, Noga (1994) included low salinity, temperature, dissolved oxygen and pH as environmental risk factors that can induce skin injury and which eventually could lead to ulcerative mycosis outbreaks in estuarine environment. Data from his field and tank studies apparently support this. Moreover, a highly toxic dinoflagellate, *Pfeisteria piscicida*, was identified as a cause of skin erosions when fish are exposed to sublethal levels of the microalga. Such skin damage is sufficient for pathogens to invade fish tissue, often leading to deep ulcers (Burkholder *et al.* 1992; Noga *et al.* 1993). However, this explanation has been fiercely contested by Dykstra and Kane (2000), and therefore, the sufficient causes of UM remain unresolved.

1.1.3 Clinical signs and histopathology of EUS lesions

Diagnosis of EUS is mainly based on clinical and pathological features. Lilley *et al.* (1992) and Roberts *et al.* (1994) listed loss of appetite and sluggish movements, and affected fish usually swimming with the head out of the water as some of the behavioural signs of EUS. Gross signs include small red spots or skin erosions that develop into large ulcers, loss of scales, oedema and inflammation.

However, confirmation of EUS is highly dependent on histopathological examinations that usually show the presence of typical fungal granulomas.

The histopathology of lesions had been described for MG (Miyazaki and Egusa 1972, 1973a, 1973b, 1973 c), RSD (McKenzie and Hall 1976; Rodgers and Burke 1981; Callinan *et al.*1989), UM (Noga and Dykstra 1986) and EUS (Roberts *et al.* 1993; Chinabut *et al.* 1995; Chinabut and Roberts 1999). Principal histopathological features of EUS include the presence in the musculature of invasive, broad, non-septate fungal hyphae enclosed in layers of epithelioid cells forming granulomas, the surrounding tissue being oedematous and infiltrated by inflammatory cells and the muscle fibers undergoing degeneration and necrosis. In contrast, in other infections of fish by Saprolegniales such as saprolegniasis of salmonids and winter mortality of channel catfish, there is usually limited or no inflammatory or granulomatous tissue reactions (Puckridge *et al.* 1989; Miyazaki 1994). Consequently these reactive lesions are the main pathological attributes of EUS although slight variations may be noticed depending on the affected species and environmental conditions.

Chinabut and Roberts (1999) classified EUS lesions into five types, according to clinical and histopathological features. They also based these types on the lesions exhibited by EUS-susceptible species and on fish species refractory to the fungal infection. Callinan *et al.* (1989) described two main lesions in sea mullet: erythematous dermatitis which is characterised by lesions of chronic dermatitis but without fungal elements, and necrotising dermatitis, manifested by an extensive granulomatous response due to invasion of fungal hyphae into the dermis and skeletal muscles. Erythematous dermatitis lesions and some intermediate-type lesions with little fungal invasion usually heal, while necrotising dermatitis lesions progress into dermal ulcers. These latter lesions may eventually heal by skin and scale repair, fibroplasia, removal of dead muscle tissue and regeneration of myofibres.

Similarly, Noga and Dykstra (1986) classified early UM lesions into two types. They are either small, flat red areas (type I) or raised lumps (type II) on the flank. These early lesions will eventually advance to deep ulcers involving the

underlying muscle. Atlantic menhaden usually exhibit intense chronic inflammatory response with mycotic granuloma formation attributed to macrophages and epithelioid cell activity. Although they are present in both lesion types, fungal granulomas in type I lesions are not as distinct and well developed as those observed in type II lesions. The fungi can also invade internal organs with a resultant granulomatous response.

In Japan, where the first case of aphanomycosis was reported in 1971 among cultured ayu, *Plecoglossus altiveles*, goldfish, *Carassius auratus*, bluegill, *Lepomis macrochirus,* and wild fish, the common histopathological signs were mycotic granulomas in the dermis, skeletal muscles and visceral organs (Miyazaki, 1994). Affected muscle fibres were necrotic with inflammatory cell infiltration and hemorrhages. Wada *et al.* (1994) also reported two types of lesions that developed in dwarf gourami, *Colisa lalia*. These were based on the gross appearance and pathological features.

1.1.4 Treatment and/or prevention of EUS

The control and treatment of EUS in wild fishes is not feasible but effective preventive measures and prophylactic procedures can be applied in farmed species to minimise losses. At present, application of lime to ponds seems effective (Lilley *et al.* 1992; Ahmed and Rab 1995) since it can improve water quality, specifically, alkalinity and water hardness. However, application of keiserite ($MgSO_4$) to offset variations in alkalinity and water hardness did not prevent EUS outbreaks in experimental rice-fish plots (Lumanlan-Mayo *et al.* 1997) so the efficacy of lime may be attributable to some other effect. Other chemical treatments include common salt or table salt (NaCl), potassium permanganate ($KMnO_4$), formalin, malachite green, bleaching powder and antibiotics (Lilley *et al.* 1992), which can control different pathogens associated with EUS. Callinan (undated) tested a chelated copper (Coptrol), which was found to be effective to control EUS outbreaks in ponds and rice-fish plots. Campbell *et al.* (1998) trialled some fungicides, plant and other natural products and extracts to identify substances with possible anti-*Aphanomyces* properties. Apparently, the tested substances were more effective when mixed with

surfactants. Understanding the pathogenesis of a multifactorial disease like EUS would certainly be helpful in identifying appropriate preventive measures, which will be both effective and environmentally acceptable.

1.1.5 Experimental EUS

In 1994, a regional seminar on EUS sponsored by the British Overseas Development Administration was held in Bangkok in order to evaluate the status of the disease, the progress of research regarding its causes and to provide directions for future research. At the end of the seminar, a definition of EUS was agreed upon as follows: "EUS is a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious aetiology characterised by the presence of invasive *Aphanomyces* infection and necrotising ulcerative lesions typically leading to a granulomatous response" (ODA 1994). Based on this definition and on some recent findings, although *A. invadans* may not be the only pathogen involved in most EUS lesions, it is apparent that this fungus plays the most significant role in EUS outbreaks.

At present, *Aphanomyces* infection can be reproduced by injection of motile zoospores or hyphae into susceptible species. Hatai (1994) injected healthy goldfish with zoospores of the pathogenic *Aphanomyces* isolated from goldfish *Carassius auratus*, and was able to produce typical granulomas in the muscle, kidney, spinal cord and other organs. In the same report, intramuscular injection of *A. piscicida* hyphae isolated from ayu, *Plecoglossus altiveles*, showed that 11 fish species exhibited different degrees of susceptibility to the fungus. Roberts *et al.* (1993) also induced typical EUS lesions by inserting some mycelia under the incised dermis in snakehead, *Channa striata.*. Chinabut *et al.* (1995) injected snakehead fish with zoospore suspensions from an *A. invadans* isolate to examine the effect of different temperatures on the response of snakehead fish to the fungus. Khan *et al.* (1998) also used intramuscular injections of *A. invadans* zoospores to investigate the susceptibility of five fish species.

Other approaches, not involving injection of fungal elements, have been used by Callinan and co-workers. Fraser and Callinan (1995) reproduced the chronic

inflammatory and mycotic granulomatous response in mullet, *Mugil cephalus*, by using skin abrasion followed by exposurer to *A. invadans* zoospores. Callinan (1997) exposed sand whiting, *Sillago ciliata*, to both acid sulfate soil runoff (pH 3.1 and 5.1) and artificially reconstituted water that approximated the qualities of acid sulfate soil runoff and these fish were subsequently placed in water with zoospores. The fish developed lesions typical of EUS on day 6-postexposure. These findings suggest that an initial skin insult is necessary prior to *A. invadans* spore attachment/invasion.

Reproduction of EUS under controlled conditions proved to be very important in demonstrating the pathogenic role of *A. invadans* and the accessory role of predisposing factors like the rhabdoviruses isolated from infected fish in Thailand, as reported recently (Kanchanakhan *et al.* 1999), and acid soil runoff in Australia which can lead to debilitation of the fish's skin. Different biological and environmental factors have been associated with EUS outbreaks in various countries in the Asia-Pacific region. In the Philippines and in some other countries, the full pathogenesis of EUS still remains to be elucidated. Results from the NACA Regional Research Programme on Relationships between EUS in Fish and the Environment investigations (Bondad-Reantaso *et al.* 1992; Phillips 1994) and the ACIAR-funded collaborative project (Callinan undated) showed low and diurnal fluctuation of water temperature as consistent factors in EUS outbreaks. To help elucidate these observations, Chinabut *et al.* (1995) investigated the differential histopathological response of snakehead to *A. invadans* infection when fish were held at varying temperatures. However, the full role of temperature in the pathogenesis of EUS lesions is yet to be understood.

1.2 Temperature and Defense Mechanisms in Fish

Fish, like any other ectotherms, are always affected by changes in environmental temperature and thus, disease processes and defense mechanisms in fish are dependent on the prevailing water temperature. Also, temperature can influence not only the protective mechanisms of the host but also the capability of potential pathogens to infect and survive (Finn and Nielsen 1971).

1.2.1 Effects of temperature on the skin of fish

Fish skin is one of the main barriers between the fish and its environment. This barrier is composed of a mucous coat and epidermal cell layers, the number of which varies from species to species, that serve primarily as protection against invasion of foreign organisms and harmful substances. However, since the skin is subjected continuously to various conditions in the aquatic environment, it is always likely that structural and physiological changes that can compromise its protective function could occur.

An important disease of channel catfish, *Ictalurus punctatus*, called "winter saprolegniasis" is now understood to be related to a rapid drop in water temperature. Apparently, the epidermal mucous cell frequency decreases considerably three days after a drop in temperature (Quiniou *et al.* 1998). The number of mucous cells returned to normal levels after 6 days, but when fish were exposed to *Saprolegnia* spores, the mucous cells continued to decrease until the fish died from fungal infection. This suggests that once *Saprolegnia* infects the skin at low temperatures, the normal renewal of mucous cells is inhibited and a vicious cycle of continued infection and reduced mucous cell population is established.

Some of the earliest investigations on the seasonal changes on epidermal thickness and number of mucous cells in salmonids were undertaken largely due to occurrences of *Saprolegnia* infections during the abrasive spawning period. In brown trout, *Salmo trutta,* Pickering (1977) reported cyclical changes in the epidermal thickness in both male and female fish during consecutive spawning activities from December to January, with male trout epidermis always thicker than in females. In addition, skin mucous cells decreased during winter in mature male brown trout. This was also reported in Atlantic salmon, *Salmo salar* (Wilkins and Jancsar 1979). Burton and Fletcher (1983) also found marked seasonal variations in the epidermal thickness and concentration of mucus-secreting goblet cells and epidermal melanophores in winter flounder, *Pseudopleuronectes americanus*. The reduction in mucous cells, hence reduced

mucus production as well, increases the susceptibility of fish to fungal invasion (Cross and Willoughby 1989).

Wood *et al.* (1986; 1988) studied the attachment to, and interaction of spores of saprophytic and pathogenic *Saprolegnia* with, the mucus of brown trout, *S. trutta*. Spores of both fungi accumulated to the same degree on the mucus, and were continuously shed with the mucus. However, some of the pathogenic propagules were retained on the fish skin whereas those of the saprophytic strain were not. It was suggested that the persistence of the pathogen was countered by the presence of an inhibitory substance in the mucus and it was also noted that some fish cells, presumably neutrophils or lymphocytes, were attached to some hyphae which had developed from spores in the mucus of the challenged fish. Hence, it appears that the defense mechanism of the fish against *Saprolegnia* is made up of three components: physical removal of spores through shedding of mucus, an inhibitory substance in the mucus and a cellular response in the mucus, which eventually lysed the hyphae.

1.2.2 Effects of temperature on the immune system of fish

The effects of low temperature stress on the immune function of fish have been well studied and have mainly focused on the mechanisms of the immune suppression, which is often induced. In general, some immune responses are inhibited while others are delayed. According to Bly and Clem (1996), immune functions, which include mitogen-induced T lymphocyte proliferation, mixed leukocyte reactions, primary antibody production to thymus-dependent antigens, macrophage-activating factor production by T cells, respiratory burst activity in phagocytes as well as complement and C-reactive protein activities, are inhibited by low temperatures. Immune parameters that are slowed include antibody production due to thymus-independent antigens, chemotaxis, phagocytosis, interleukin-1 secretion, ligand binding to cell surface receptors and lateral transport of receptor-ligand complexes across the cellular surface.

In terms of specific immune response, in his work on antibody production in carp, Avtalion (1969) reported that at 12 °C, no circulating antibodies were detected in

bovine serum albumin (BSA)-immunised fish. However, antibody production occurred at low temperature if the fish were initially immunised at 25 °C, which indicates that fish can still produce antibodies at low temperature and they have the capacity for immunological memory. This response was also reported in channel catfish immunised against *Edwardsiella ictaluri* (Plumb *et al.* 1986). In other studies, it was shown that low temperature mainly delays the peak response antibody production (Paterson and Fryer 1974; Rijkers *et al.* 1980; Groberg *et al.* 1983; Stolen *et al.* 1984; Ahne 1986). Juvenile summer flounder, *Paralichthys dentatus*, were reported to be more susceptible to *Trypanoplasma bullocki* infection at low temperature but humoral immunity can destroy and eliminate the parasites when temperature increases in spring (Sypek and Burreson 1983; Burreson and Frizzell 1986). Moreover, Suzuki *et al.* (1997) reported that skin mucus IgM of rainbow trout was low during periods of low temperature, a condition that likely occurs during the spawning season when the fish are susceptible to fungal infection.

To explain the mechanism of immunosuppression caused by low temperature in fish, a series of experiments were undertaken using channel catfish. In 1984, Miller and Clem reported the different primary response of leukocytes to thymus-dependent and thymus-independent antigens, with the *in vitro* reaction against soluble thymus-dependent antigen being inhibited at incubation temperatures of 22 °C and 17 °C compared to those incubated at 27 °C and 32 °C with cells obtained from fish acclimated at 27 °C. This showed that the two subpopulations of lymphocytes had differential temperature tolerance, with, presumably, T cells (virgin T cells) being more sensitive to lower temperatures. Bly et al. (1986) investigated the binding of concanavalin A at different *in vitro* temperatures (0.5 °C, non-physiological for channel catfish; 12 °C and 17 °C, physiological but non-permissive for T cell mitogen response; 22 °C, 27 °C and 32 °C, physiological and permissive) and showed that T-cell proliferation was inhibited at low temperature (non-permissive) even though concanavalin A binding occurred at all temperatures. In order to identify the mechanism involved, these authors undertook a series of experiments which showed that even though mitogen binding to the cell surface occurred at all temperatures used and the surface antigen capping of T lymphocytes is not sensitive to low temperature (Bly *et al.*

1988), the key mechanism is that B cell membranes undergo homeoviscous adaptation at non-permissive temperature more readily than T cells. Apparently, 3-5 weeks is required for the T cells to undergo increased membrane fluidity against 1-3 weeks for B cells. Biological membranes adapt to low temperature by increasing their fluidity, made possible by an increase in the unsaturated to saturated fatty acid ratio in the phospholipid component (Cossins 1977; Bell *et al.* 1986). The viscosity of the membrane can affect cell membrane receptors, receptor binding affinities, cell-cell interactions, permeability and enzyme activities (Blazer 1991; Obach and Baudin Laurencin 1992; Kiron *et al.* 1995; Lingenfelser *et al.* 1995) hence its importance in the fish immune response. Bly and Clem (1992) reported that channel catfish B cell membrane could rapidly increase the stearic acid:oleic acid ratio, hence increasing membrane fluidity while T cells accumulated stearic acid after stimulation with mitogens. These alterations in the plasma membrane fatty acids of lymphocytes occurred as an adaptation to non-permissive temperatures. These changes, specifically an increase in oleic acid: stearic acid ratio, were reported to have facilitated helper T cell function at nonpermissive temperature.

Research on the influence of low temperature on nonspecific immunity is not as extensive as that on specific immunity, and the results are at times contradictory. However, most reports agree that nonspecific immune responses activities play a significant role during periods of low temperature when specific immunity is impaired. Phagocytosis, either by polymorphonuclear neutrophil (PMN) or mononuclear phagocytes (macrophages or monocytes), is an important function in the inflammatory process and the eventual destruction of pathogens.

Finn and Nielsen (1971) reported the effects of temperature on rainbow trout, *Oncorhynchus mykiss*, injected with either complete Freund's adjuvant or a suspension of killed staphylococci and held at 5 °C or 15 °C. There was no qualitative difference in the response but there was a delay in the inflammatory response in fish at lower temperature. In a study on the artificial infestation of the skin of plaice, *Pleuronectes platessa*, with metacercariae of the digenean, *Cryptocotyle lingua,* epidermal lesions developed with myofibrillar degeneration

and inflammation. However, the formation of parasite cysts and encapsulation by the host was remarkably slower at 5 °C than at 15 °C (McQueen *et al.* 1973).

Generally, *in vitro* studies on phagocytosis at low temperature showed both PMN and macrophage have the capacity to adapt to such temperature stress. Although O'Neill (1985) reported that phagocytosis in brown trout PMN was optimal at 20 °C and was reduced at lower temperature, Angelidis *et al.* (1988) and Le Morvan *et al.* (1997) showed that in sea bass and carp, respectively, head kidney cells gave a higher chemiluminescence response (as a measure of phagocyte activity) at lower temperature, even though it took a longer time to reach the peak response. However, Ainsworth *et al.* (1991) and Dexiang and Ainsworth (1991), suggested that phagocytes are more resistant to decreasing temperature than lymphocytes since there was only partial suppression or reduced phagocytic activity at 10 °C. In addition, they reported adaptation of respiratory burst due to acclimation at low temperature. Thus, it seems that phagocytes can recover rapidly from temperature-induced functional depression.

Blood granulocytes from tench, *Tinca tinca*, were also reportedly capable of adapting to winter temperatures, by exhibiting greater phagocytic activity (Collazos *et al.* 1994a). Additional work, however, showed that phagocytic function was seasonal, that is, activity was higher during winter (12 °C) and summer (30 °C) compared to autumn and spring when average temperature is 22 °C (Collazos *et al.* 1994b). In 1996, Collazos *et al.* also reported seasonal difference with respect to the response of lymphocytes stimulated with mitogens. Lymphocyte proliferative responses were higher in summer than at winter, which suggests immunosuppression at low temperature. Similarly, in gilthead sea bream, *Sparus auratus* (Tort *et al.* 1998), nonspecific immune indices, which include serum complement activity, erythrocyte agglutination, lysozyme levels, lymphocyte counts and macrophage respiratory burst activity, were found to be depressed during winter. *In vitro* and *in vivo* study on rainbow trout leucocytes (Hardie *et al.* 1994) showed that production of the macrophage activating factor (MAF) was temperature sensitive. However, macrophages kept at low temperature remained reactive to exogenous MAF and even showed an increase

in respiratory burst activity. They further proposed that providing stimulants directly to macrophages could mitigate the inhibitory effect of temperature on MAF production of T cells.

To further investigate the interaction of temperature and fish immune responses, Bly and Clem (1991) subjected channel catfish to an abrupt change in water temperature. Results indicated that a temperature decrease from 23 °C to 11 °C suppressed both B and T cell responses. In 1993b, Bly *et al.* reproduced "winter saprolegniasis" in a laboratory model by subjecting channel catfish to an abrupt decrease in water temperature and to *Saprolegnia* zoospores. Bly *et al.* (1993a) confirmed through histopathology that at low temperature, the infected fish were immunocompromised as revealed by a lack of inflammatory response as compared with fish kept at 24 °C that actively mounted a cellular defense against *Saprolegnia.* Serum antibodies were not detected and phosphorylcholine-reactive protein, which appears to be similar to human C-reactive protein, was also minimal during spells of rapid temperature decrease (Szalai *et al.* 1994). Thus, this low temperature-induced immunosuppression along with the decrease in mucous cell density and mucus production in channel catfish make them susceptible to fungal infection during winter.

1.3 Rationale for this study

While seasonal occurrence of EUS is well documented in the Philippines and in some other Asian countries, the underlying pathogenesis of the disease has not been elucidated. It was against this background that this study was undertaken.

In a technical handbook on EUS, Lilley et al. (1998) proposed that most disease outbreaks take place due to the combined effects of a number of causal factors. The authors presented the multifactorial causation of EUS by using pie diagrams, with each slice representing a component cause or factor. Combinations of such causal factors usually lead to disease and collectively known as a "sufficient cause". It should be emphasised that various combinations of component causes may comprise the sufficient cause of a disease, however, all sufficient causes of a

particular disease have a "necessary" cause, which is always present for the disease to occur. Figure 1.1a shows a natural sufficient cause for EUS while fig.1.1b represents an experimental sufficient cause for EUS. In both diagrams, the "necessary" cause is the fungal pathogen, *A. invadans*.

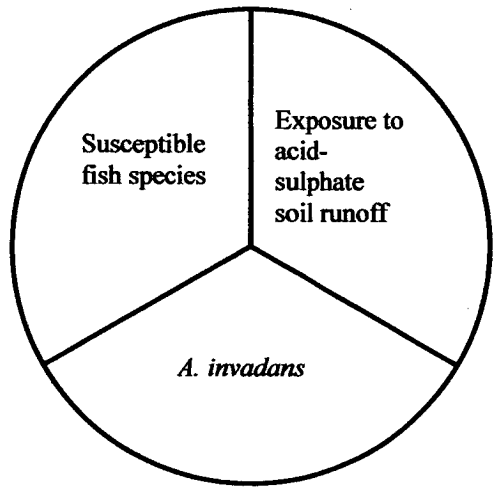


Fig.1.1a Natural sufficient cause of EUS, exemplified by outbreaks in NSW, Australia.

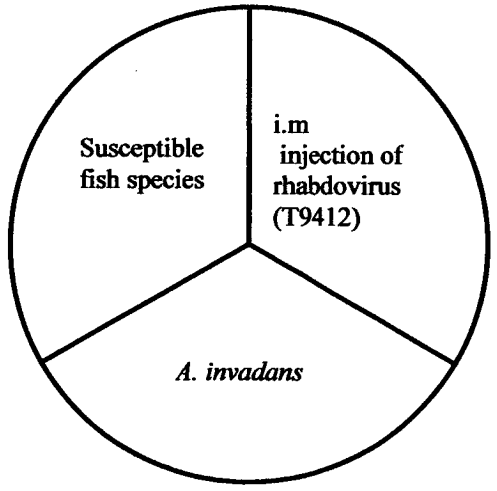


Fig. 1.1b Experimental sufficient cause of EUS performed using a rhabdovirus isolated from EUS-affected fish from Thailand.

Figure 1.1 Pie diagrams of sufficient causes of EUS.

It should be emphasised that low and/or fluctuating temperature is the only consistent factor that could be related to EUS outbreaks in the Philippines, based on published evidence. In the only published report of EUS occurrence in estuarine conditions in the Philippines, pH ranged from 7.1-7.5 during an outbreak which affected several fish species (Callinan *et al.* 1995b). Thus, while low pH due to acid runoff is a sufficient cause of EUS in Australia, such condition has not been adequately shown as an important factor in EUS outbreaks in Philippines. Consequently, a proposed sufficient cause of EUS occurrence in the Philippines would be shown by fig. 1.2, with low and/or fluctuating temperatures and susceptible fish as component causes while *A. invadans* is still the necessary cause.

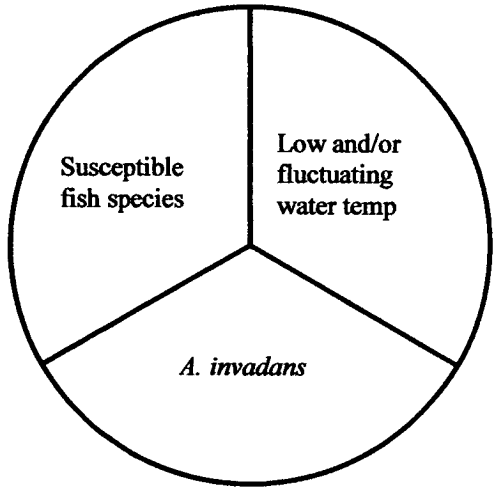


Figure 1.2 Proposed sufficient cause of EUS in the Philippines

Therefore, the main thrust of this project was to test the hypothesis that low and/or fluctuating water temperatures are a sufficient cause of EUS in the Philippines. Also, research was undertaken to identify treatments or prophylactics which might ameliorate the effects of temperature stress on fish exposed to, or infected with, *A. invadans*.

The fact that this study was performed in Tasmania had its advantages and disadvantages. The absence of classical EUS meant that there was no threat of natural infection perturbing the study, but because the disease was exotic, all experiments had to be conducted under strict quarantine. Also, susceptible fish species were not readily available in the State and only some of those suitable for the study were permitted to be imported. On the other hand, the availability of temperature-controlled facilities at the Key Centre for Aquaculture meant that studies on the effects of low and/or fluctuating temperatures could be conducted at any time of the year, which was a distinct advantage.

Chapter Two

Comparative histopathology of three-spot gourami, *Trichogaster trichopterus* Pallas, and sand whiting, *Sillago ciliata* Cuvier, experimentally infected with *Aphanomyces invadans* spores

2.1 INTRODUCTION

Epizootic ulcerative syndrome (EUS) affects a wide range of freshwater and brackishwater fish species. The consistent histopathological features of the skin lesions include the presence of intense inflammatory cell infiltration and the formation of granulomas due to the invasion of the pathogenic fungus, *Aphanomyces invadans* (Callinan *et al.* 1989; Wada *et al.* 1994; Chinabut and Roberts 1999). Nonetheless, some differences in the clinical and pathological responses to the fungal infection were detected by these authors, which could be primarily attributed to the degree of susceptibility/resistance of the hosts to the pathogen.

Experimental induction of EUS lesions in various fish species had been undertaken either through intramuscular injections of fish with viable zoospores or small pieces of fungal hyphae, or by exposure of scarified fish to spores (Hatai *et al.* 1984; Roberts *et al.* 1993; Callinan 1994b). Based on fish mortality, Hatai (1994) demonstrated that "tairiku baratanago" or *Rhodeus ocellatus* was highly susceptible while some fish like the ayu (*Plecoglossus altiveles*), bluegill (*Lepomis macrochirus*), crucian carp (*Carassius carassius)* and goldfish (*Carassius auratus*) were classed as susceptible. Species that were classified as less susceptible were the rudd (*Scardinius erythrophthalmus*), and rainbow trout (*Oncorhynchus mykiss*) while carp (*Cyprinus carpio*), eel (*Anguilla japonica*), loach (*Misgurnus anguillicausatus*) and catfish (*Parasilurus asotus*) were resistant. However, Khan *et al.* (1998) used progressive histopathological changes in tilapia (*Oreochromis niloticus*), rosy barb (*Puntius schwanenfeldi*), rainbow trout (*Oncorhynchus mykiss*), roach (*Rutilus rutilus*) and stickleback (*Gasterosteus aculeatus*) injected with *A. invadans* spores to more precisely demonstrate differential host susceptibility to the pathogenic fungus.

As the influence of various biological and environmental factors on the pathogenesis of the disease are yet to be fully established, a standardised method of reproducing EUS under controlled condition is required when undertaking trials to demonstrate the relative susceptibility of different fish species to

A. invadans. In this chapter, two separate trials were undertaken to develop a model of the pathology of EUS. The three-spot gourami (*Trichogaster trichopterus*) and sand whiting (*Sillago ciliata*), two fish species reported to be susceptible to EUS were used to assess the response of each species qualitatively and quantitatively based on the sequential pathological changes produced by injection of zoospores of *A. invadans*. The three-spot gourami is an obligate air-breathing fish at temperatures from 20-25 °C (Burggren 1979) found in the lakes, swamps, rice paddies, rivers and streams of Southeast Asia, Sumatra and Borneo (Pollak *et al.* 1981). Sand whiting are benthic estuarine species, distributed from Papua New Guinea to Tasmania, but commonly found in northern New South Wales and southern Queensland (Mckay 1985, cited by Burchmore *et al.* 1988).

2.2 MATERIALS AND METHODS

2.2.1 Fish and husbandry conditions

Sixty (60) three-spot gourami (mean total length 6.6 ± 0.85 cm, bodyweight 3.0-5.0 g) obtained from a local pet shop were acclimated in 2 x 40 L glass tanks (30 fish per tank) with undergravel filtration. The same number of juvenile sand whiting (mean weight 3.6 ± 0.71 g) from the Bribie Island Aquaculture Research Centre (DPI Queensland, Australia) was similarly acclimated in glass tanks for one week (30 fish x 2 tanks). The 3-spot gouramis were fed twice daily with a commercial fish flakes while the sand whiting were fed at 1.5% body weight with a formulated crumbled, low fish oil (4.5%) diet. Immersion heaters were used to maintain water temperature at 26–27 °C. For the sand whiting, salinity ranged from 9-12‰ while the 3-spot gouramis were held in fresh water. Partial water change was undertaken every day using dechlorinated/aged municipal water for the gouramis and seawater (Weymouth, Tasmania) diluted with municipal water for the sand whiting.

2.2.2 Production of fungal zoospores

Blocks (approx. 0.5 cm^3) of *A. invadans* hyphae growing on glucose yeast agar (GYA) were aseptically cut and placed in glucose yeast broth (3 blocks x 100 ml) to grow fungal mats. After 4 days, each fungal mat was washed 5 times in autoclaved distilled water, placed in a Petri dish with autoclaved pond water (from a farm dam at Glengarry, Tasmania) and incubated at 20 °C for sporulation. After 24 hours, motile zoospores were estimated for each plate using a Sedgewick-Rafter counting chamber under a light microscope (Appendix 1).

Isolate 24P was used in all the experiments undertaken for this research. This was isolated from a sea mullet caught from Saltwater Creek, Townsville, Queensland in May 1990. This fungal isolate was passaged a couple of times in sand whiting and reisolated to recover its pathogenicity. However, maintenance of *A. invadans* isolates at the RVL Wollongbar, NSW was stopped when the ACIAR-funded EUS collaborative project was terminated in 1996, thus there was no reference strain to compare it with. In fact, in one occasion when the culture was lost during the research, it had to be imported from Scotland. On several occasions, attempts were made to obtain fresh isolates from EUS outbreaks in NWS and Queensland but were all unsuccessful.

2.2.3 Fish inoculation

Fungal spore inocula were prepared by diluting the original suspension to 10^3 spores mL^{-1} concentration. This was based on preliminary trials (Appendix 1) conducted to induce EUS lesions in both species. Three-spot gouramis were anesthetised with 80 mgL^{-1} benzocaine and 30 fish were injected intramuscularly (Fig.2.1a) with 0.05 ml of 1.7 x 10^3 zoospore mL^{-1} suspension while the other 30 fish (control) were injected with 0.05 mL sterile phosphate-buffered saline (PBS, pH 7.1). The sand whiting (51 fish) which remained after the acclimation period were anesthetised with 60 mgL^{-1} benzocaine: 27 fish were injected with 0.05 mL of 1.3 x 10^3 zoospore mL^{-1} suspension and 24 fish (control) were injected with sterile PBS. The fish were allowed to recover in clean, aerated water after the inoculation and placed in their respective experimental tanks.

2.2.4 Sampling and experimental conditions

Daily feeding was resumed a day after inoculation. Water temperature and dissolved oxygen were measured daily using WTW Oxi 96 Oxical-S® Oximeter while alkalinity, pH and NH_3-N levels were checked once (3-spot gouramis) and twice (sand whiting) during the experiment using Aquasonic® test kits. Salinity was monitored daily (sand whiting only) using an Iwaki® hand refractometer. One-half of the water in each tank was replaced daily, as such no significant change in either alkalinity, pH and NH_3-N levels were expected during the trials, and were thus measured only once.

For the 3-spot gourami trial, mean daily water temperature in the control tank was 26.3 ± 0.7 °C and 27.0 ± 0.78 °C for the zoospore-inoculated group. Mean daily DO were 7.5 ± 0.92 mgL^{-1} and 6.6 ± 0.90 mgL^{-1} for the control and zoospore-inoculated group, respectively. Alkalinity was 50 mgL^{-1} $CaCO_3$ and NH_3-N was <0.1 mgL^{-1} for both groups. For the sand whiting, mean daily temperature were 26.3 ± 0.27 °C and 26.1 ± 0.70 °C for the control and zoospore-inoculated fish, respectively. Mean daily DO was 5.07 ± 0.97 mgL^{-1} for the control and 4.88 ± 0.68 mgL^{-1} for the zoospore-inoculated fish. Salinity ranged from 6-8 ‰; alkalinity was 75 mgL^{-1} $CaCO_3$, pH was 7.2-7.5 and NH_3-H was < 0.1 mgL^{-1} for both tanks.

Three fish each from PBS- and zoospore-injected fish were euthanased with 200 mgl^{-1} benzocaine, at 24-hr, 2, 4, 6, 8, 10 and 12 days post inoculation for histopathological examination. Mortalities and gross signs of fungal infection were monitored daily.

2.2.5 Histopathology

Tissue samples were fixed in 10% neutral buffered formalin, trimmed and decalcified (Fastcal®, Histo•Labs Pty Ltd), dehydrated in graded series of ethanol, embedded in paraffin, sectioned at 4-5 μm and stained using the periodic acid-Schiff (PAS) technique. The sections were qualitatively and quantitatively

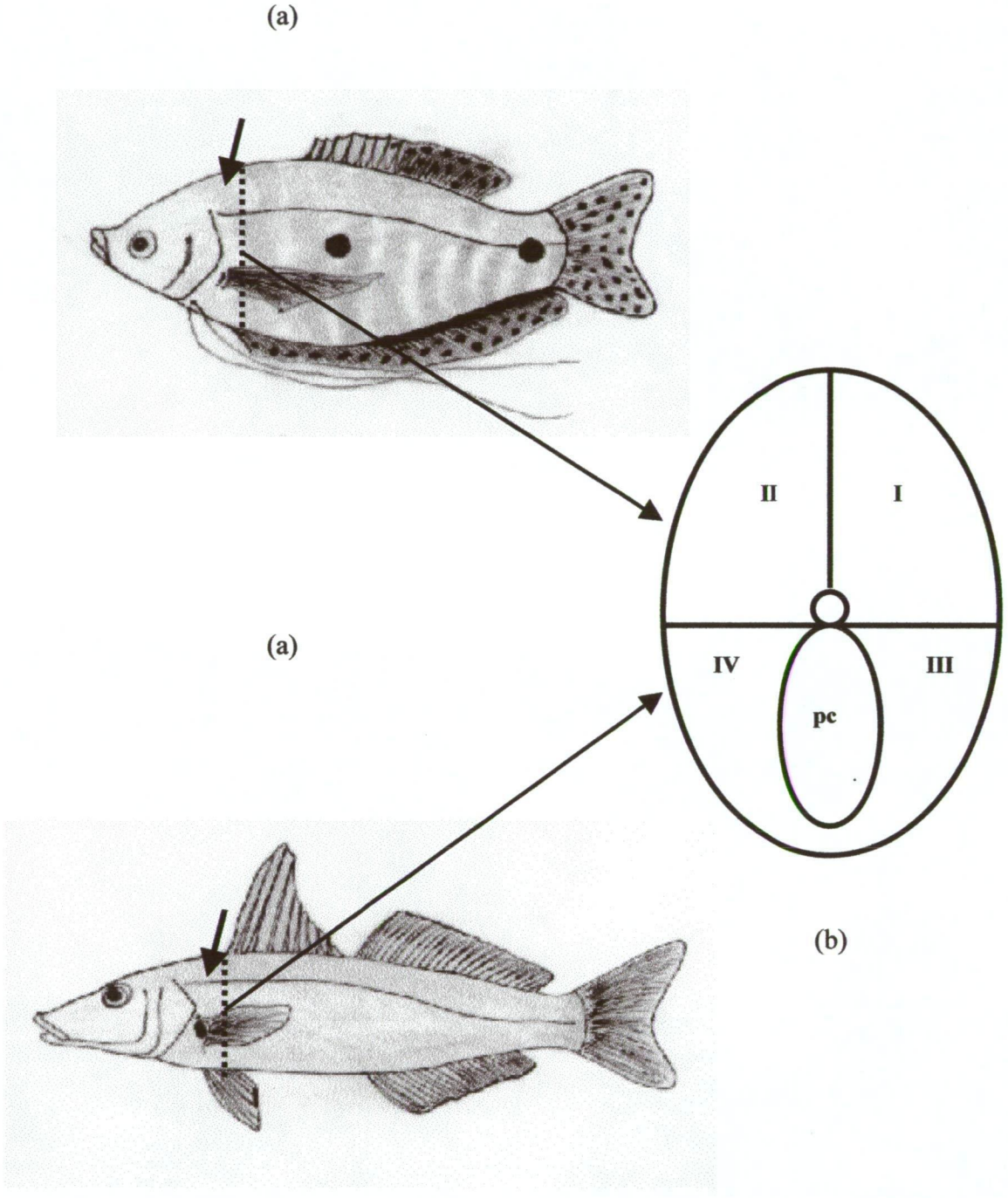


Figure 2.1 (a) Injection sites (arrow) in experimental fish. (b) Diagram of a fish's cross-section showing the four quadrants where sites for quantitative analyses were randomly selected. (I – Quadrant 1; II- Quadrant 2; III-Quadrant 3; IV-Quadrant 4; pc- peritoneal cavity)

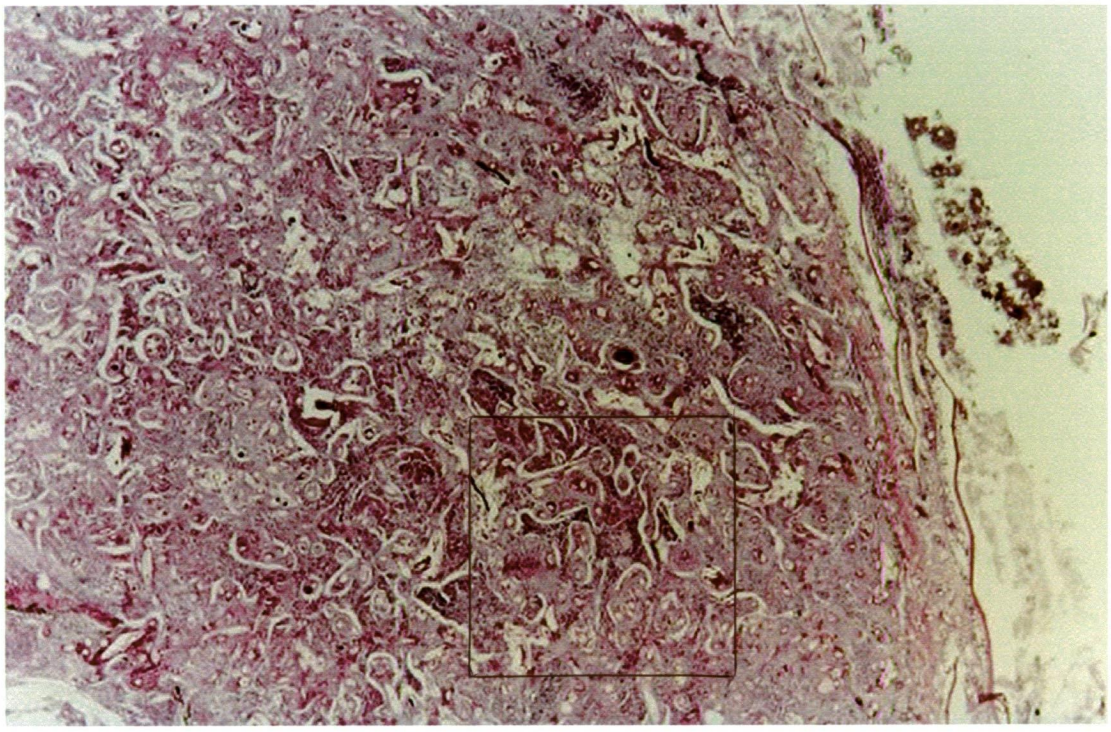


Figure 2.2 Photomicrograph of sand whiting to illustrate a randomly chosen site in Quadrant I. The □ represents 1 mm^2 area where mycotic granuloma counts were taken. Mycotic granulomas were counted from 4 areas (1 mm^2 x 4 quadrants) for each fish sample.

assessed. The qualitative sequential changes in the lesions were examined using an Olympus BH-2 light microscope. Since the most distinct pathological feature of EUS is the chronic granulomatous and inflammatory response of the host against *A. invadans*, the quantitative analyses of the sections included fungal granuloma counts and the measurement of lesion area infiltrated with inflammatory cells.

The mycotic granulomas were counted per one mm^2 in four quadrants of a cross-section of each fish, using a microscope with an eyepiece micrometer at x100 magnification. Quadrant 1 is the right, dorsal area (epaxial muscle mass), quadrant 2 is the left, dorsal portion, quadrant 3 is the right, ventral (hypaxial) area and quadrant 4 is the left, ventral part (Fig.2.1b). The data were expressed as the total mycotic granulomas in the 4 randomly chosen sites in the 4 quadrants (Fig.2.2).

The area infiltrated by inflammatory cells for each fish was measured in 4 randomly selected sites in the 4 quadrants using an Olympus Cue-2 image analyser. Epithelioid cells enveloping the fungal hyphae were not included in the measurement. The data were reported as percentage cellular infiltration which was calculated by dividing the infiltrated area of a selected site in a quadrant with the total area of that particular site, multiplied by 100.

2.3 RESULTS

Gross observations

Fish in the control groups (PBS-injected) for both species of fish did not show any behavioural or clinical signs of disease throughout the experiment. At day 2 p. i., one 3-spot gourami injected with *A. invadans* had an inflamed injection site but at day 4 p.i., most of the gouramis had swollen injection sites. By day 5 p.i., most fish had external lesions with the scales over the inflamed area being lost. Mortality started at day 7 p.i. (2 dead fish) among fish with external lesions. At

day 8 p.i., 4 gouramis died and mortality lasted until day 10 p.i.with 1 dead fish in the tank. The infected fish were lethargic and anorexic at this stage. The fish sampled at day 12 p.i. did not exhibit any external signs of infection.

Similarly, the sand whiting injected with *A. invadans* exhibited the clinical signs as the 3-spot gouramis. Most fish had lost their scales covering the injection sites at day 2 p.i. At day 4 p.i., almost all fish had highly inflamed injection sites. Petechial hemorrhages on the inflamed lesion were observed on day 5 p.i. At day 7 p.i., the infected fish were sluggish, staying at the bottom of the tank and were anorexic. Mortality first occurred at day 8 p.i.with 1 dead fish. Subsequently, 2 fish and 1 fish died on day 9 and day 11, respectively. Most of the fish were moribund on day 12 p.i. Table 2.1 shows the fish mortality incurred during the two experiments.

Table 2.1 Fish mortality during the two preliminary experiments

Days post-injection	1	2	3	4	5	6	7	8	9	10	11	12
Three-spot gourami	0	0	0	0	0	0	2	4	0	1	0	0
Sand whiting	0	0	0	0	0	0	0	1	2	0	1	0

Histopathology

A. Qualitative examination

Three-spot gourami. The epidermis and dermis (including the scales) of PBS-injected fish appeared unaffected, and very minimal tissue damage were seen in

the muscle where some muscle fibers were degenerating and there was localised hemorrhages. After 2 days p.i., inflammatory cells, mostly macrophages were tracking down along myosepta. From day 4 p.i., regenerative and reparative processes in the muscle area occurred and lasted until day 12 p.i.

For the zoospore-injected fish, there were hemorrhages and cellular infiltration mostly concentrated along myosepta and minimal myophagia 24 hr p.i. However, the epidermis, dermis and scales were unaffected. At day 2 p.i., some portion of the dermis was infiltrated with inflammatory cells, predominantly composed of macrophages and lymphocytes, but the epidermis and scales were still intact. The degree of myonecrosis and cellular infiltration had increased, even reaching the contralateral side. There was also some degree of oedema.

At day 4 p.i., some portions of the epidermis have been eroded and fungal hyphae appeared actively invading the muscle and the dermis. Generally, the hyphae were enclosed within 3-4 layers of epithelioid cells, forming mycotic granulomas. Myonecrosis was more extensive and persisted until day 6 p.i., along with severe granulomatous response and oedema (Fig. 2.3a). Granulomas were evidently thicker and fungal hyphae were present in the kidney, intestine, pancreas and the spinal cord. The dermis was inflamed with fungal granulomas. Most fungal hyphae appeared roundish and still actively invading while inflammatory cells had spread from the epaxial to the hypaxial muscles (Fig.2.3b).

At day 8 p.i., the epidermis over the lesion was lost, there was dermatitis with granuloma formations (Fig. 2.4). New blood vessels were formed in the muscle area and some granulomas have started to be surrounded with fibrous tissue, sometimes leading to linked granulomas. The hypaxial muscle mass was necrotic at this stage.

At day 10 p.i, the epidermis over the lesion was completely lost and the *stratum spongiosum* was eroded. The entire cross-section was almost completely infiltrated with inflammatory cells. The samples for day 12 p.i. did not exhibit any external signs of fungal infection as confirmed by intact epidermal and dermal layers but thick fungal granulomas were present in the muscle area.

Myonecrosis was also observed but most damaged muscle fibers had commenced regeneration.

Sand whiting. The PBS-injected fish had no significant pathological changes in the epidermis and dermis. There was some degree of cellular infiltration and degeneration of skeletal muscles at 24 hr p.i. and day 2 p.i. However, at day 4 p.i., muscle fibers appeared to be in the process of regeneration. No significant pathological changes were seen in the samples from day 6 to day 12 p.i.

In the zoospore-injected fish, the skin, including the scales were still intact 24 hr after inoculation but some muscle fibers have started to degenerate and inflammatory cells were present along the myosepta. The same signs were seen on day 2 p.i., except for some oedematous response and the presence of fungal hyphae in the epaxial muscle area (Quadrant I).

At day 4 p.i., the dermis over the main lesion was inflamed with fungal hyphae enveloped in 3-4 layers of epithelioid cells. There was myositis in the contralateral side (Quadrant II) with fungal hyphae invasion but myonecrosis was still minimal. The dermis was inflamed with some fungal hyphae at day 6 p.i. (Fig. 2.5a). However, in the muscle area, the granulomas were thicker with fibrotic periphery. At this stage, myophagia was also evident in the hypaxial muscles, along with the downward invasion of inflammatory cells.

At day 8 p.i., both the epidermis and dermis were inflamed (Fig. 2.5b) and the fungal hyphae were still actively invading unaffected muscle fibers. At days 10 and 12 p.i., the epidermis and dermis over the main lesion had been eroded or completely lost (Figs. 2.6a and 2.6b). Most of the granulomas were linked together and the extensive damage had extended to the hypaxial muscles (Quadrants III and IV).

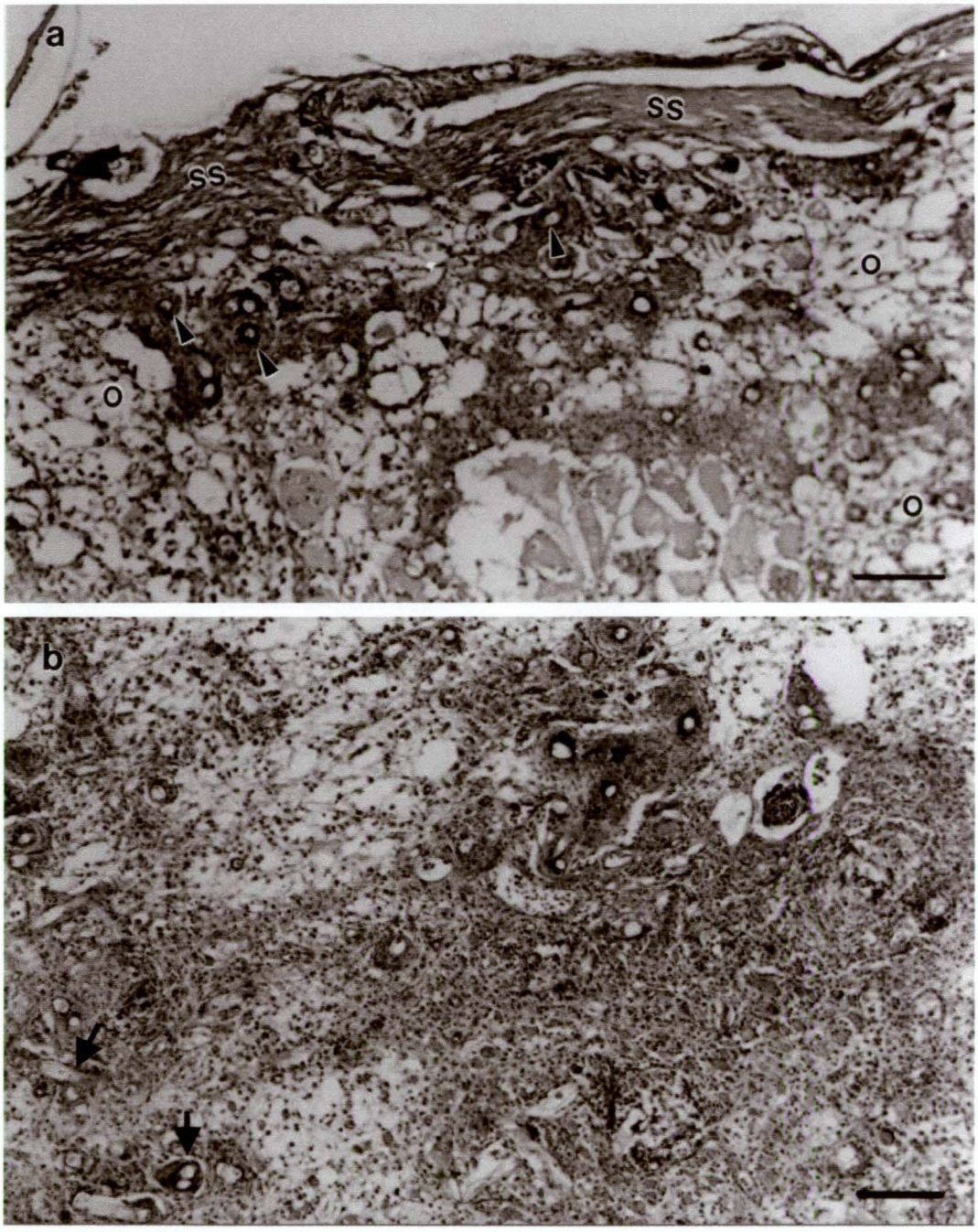


Figure 2.3 Photomicrographs of 3-spot gourami at day 6 p.i.
(a) oedematous areas (O) and mycotic granulomas (arrowheads) were evident, with the stratum compactum (SS) invaded by fungal hyphae; (b) extensive cellular infiltration and fungal hyphae invasion (arrows) in the muscle area. (PAS stain, bar = 100 μm)

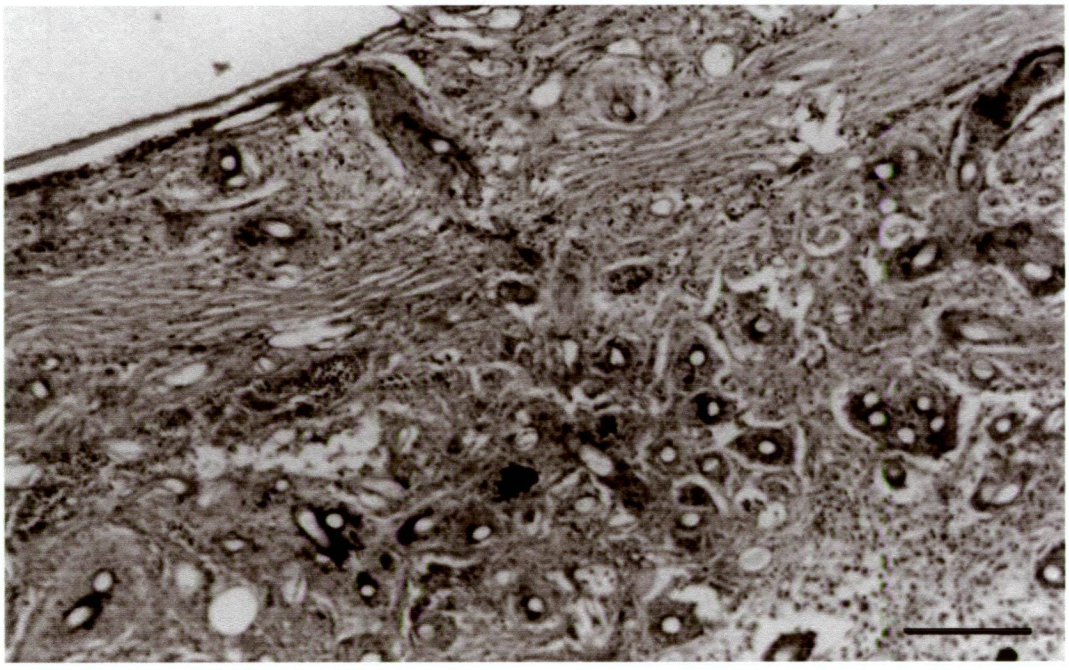


Figure 2.4 Photomicrograph of 3-spot gourami at day 8 p.i. showing a highly inflamed dermis with mycotic granulomas and fibrosis often leading to linked granulomas in the muscle area. (PAS stain, bar = 100 μm).

B. Quantitative analyses

Mycotic granuloma counts. In 3-spot gouramis, mycotic granulomas were first seen on day 4 p.i., mostly in the right and left epaxial muscle area. On day 6 p.i., all four sites examined had granulomas. There was a marked increase on day 8 p.i. but the count decreased on day 10 p.i. until day 12 p.i. In sand whiting, granulomatous response was first observed on day 2 p.i. and continued to increase on day 10 and day 12 p.i. The extent of granuloma formation was limited in the dorsal muscle mass until day 10 p.i. (Fig. 2.7).

Percentage cellular infiltration. Inflammatory cells were observed in both sides of the epaxial muscles 2 days after spore inoculation in 3-spot gouramis. At days 4 and 6 p.i., the sampled site in quadrant 1 (dorsal, right side) was markedly inflamed with macrophages and lymphocytes starting to spread ventrally. At day 8 p.i., cellular infiltrate was present in all quadrants. There was insignificant cellular infiltration on day 12 p.i. The highest total percentage of cellular infiltration was 75.2% recorded on day 6 p.i. (Fig.2.8). In the infected sand whiting, there was a marked increase in inflammatory cell infiltration on day 6 p.i., even though the infiltration was limited to the dorsal muscles. At day 8 p.i., the inflammatory cells had infiltrated the 4 quadrants. The highest total percentage of cellular infiltration was 52.8% at day 10 p.i. (Fig. 2.8).

Table 2.1 shows the percentage of cellular infiltration in 3-spot gourami and sand whiting in each quadrant. In the gouramis, quadrant 1 had the highest infiltration at day 4 and 6 p.i., then decreased dramatically on days 8 and 10 while quadrant 2 had the highest percentage at day 10. Cellular infiltrate was first measured at day 4 p.i. and at day 8 p.i. in quadrants 3 and 4, respectively. In sand whiting, there was marked infiltration in quadrant 1 at day 6 p.i. but it decreased subsequently from day 8 to day 12 p.i. In quadrant 2, cellular infiltration also increased at day 6 p.i. but continued at almost the same level until day 12 p.i. The start of inflammatory cell migration in quadrants 3 and 4 was observed at day 10 p.i. No statistical analysis was undertaken on the granuloma counts and the percentage of cellular infiltration since replicate treatment tanks were not employed in the trials.

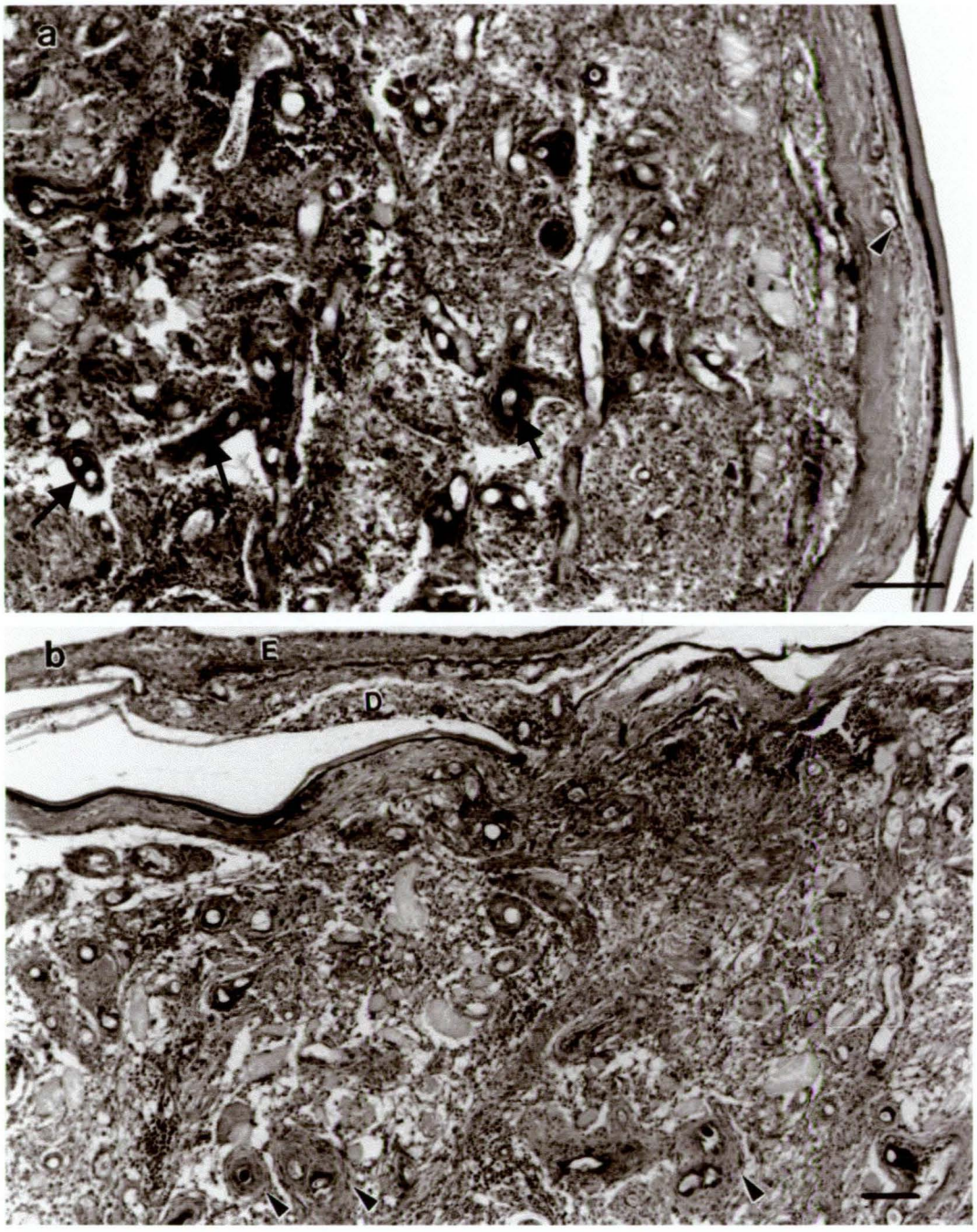


Figure 2.5 Photomicrographs of infected sand whiting. (a) At day 6 p.i., dermis was inflamed with fungal invasion (arrowhead); the mycotic granulomas in the muscle had fibrotic peripheries (arrows). (b) At day 8 p.i., the epidermis (E) was inflamed and dermis (D) was disrupted, mycotic granulomas (arrowheads) were thicker and fibrotic. (PAS stain, bar = 100 μm)

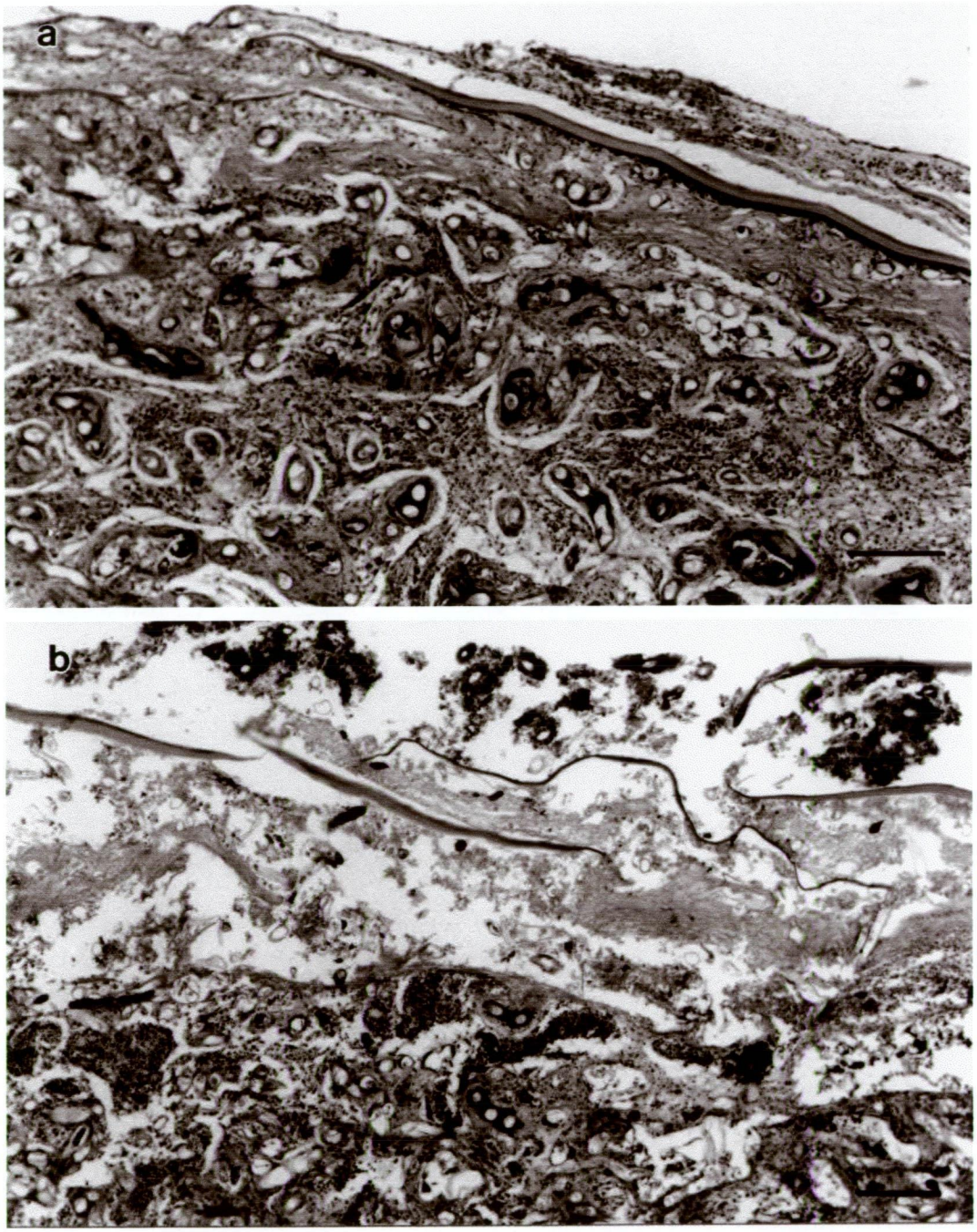


Figure 2.6 Photomicrographs of sand whiting at day 10 p.i. Skin was either eroded (a) or completely lost (b) with cellular and fungal debris attached to the exposed lesion. (PAS stain, bar = 100 μm)

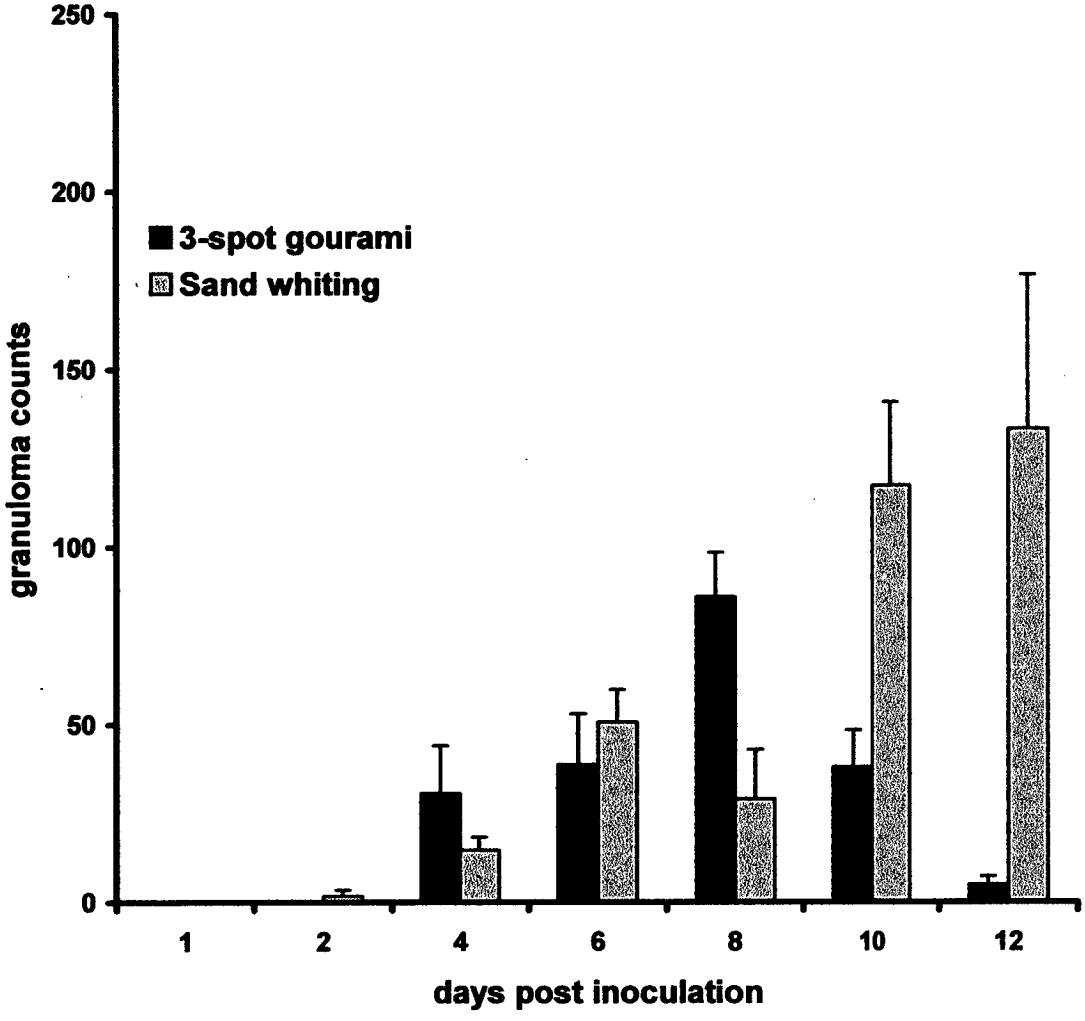


Figure 2.7 Total mycotic granuloma counts in 4 mm^2 in 3-spot gouramis and sand whiting. Bars are means ± s.e. of 3 fish at each sampling day.

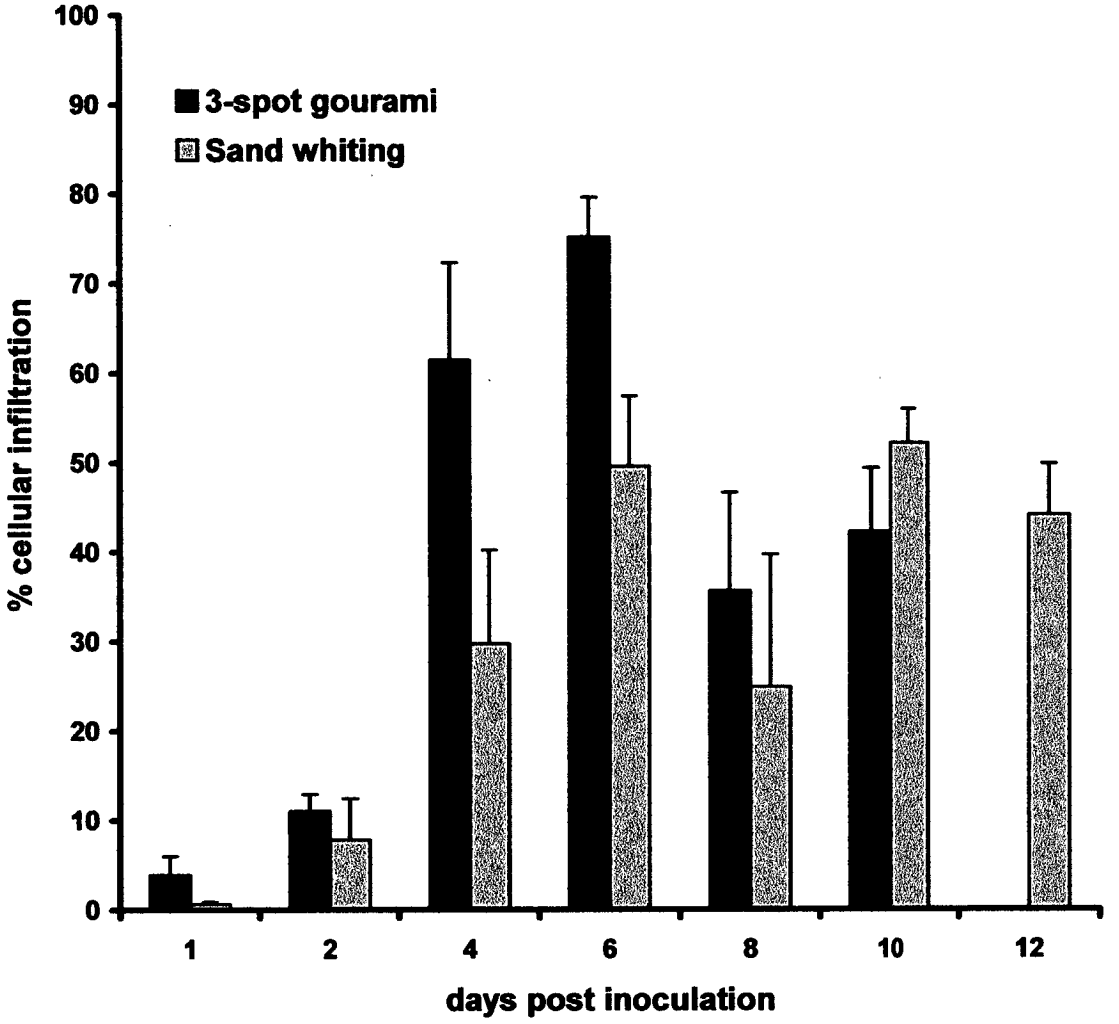


Figure 2.8 Total percentage of cellular infiltration from 4 randomly chosen sites within a cross-section of *A. invadans*-infected 3-spot gourami and sand whiting. Bars are means ± s.e. of 3 fish at each sampling day.

Table 2.2 Percentage of inflammatory cell infiltration in 3-spot gourami and sand whiting injected with *A. invadans* zoospores (Quadrant I- site of injection. Values are means from 3 fish at each sampling day.

Days Post Injection	Three-spot gourami				Sand whiting			
	Quadrant I	Quadrant II	Quadrant III	Quadrant IV	Quadrant I	Quadrant II	Quadrant III	Quadrant IV
1	2.6	1.3	0	0	0.3	0.3	0	0
2	5.8	5.2	0	0	4.7	3.2	0	0
4	54.8	5.0	1.6	0	15.5	10.2	4.0	0
6	48.4	14.9	11.9	0	33.6	15.9	0	0
8	20.5	4.5	5.6	4.9	10.4	10.1	0.4	4.0
10	16.6	23.4	1.4	0.8	13.7	12.5	8.1	17.9
12					14.1	11.9	8.9	9.3

2.4 DISCUSSION

The main histopathological features manifested by EUS-affected fish are a chronic granulomatous response and an aggressive infiltration of inflammatory cells elicited by *A. invadans* infection. Hence, aside from qualitative description of the lesions, the formation of mycotic granulomas and the inflammatory response were used as quantitative criteria to characterise the progression of EUS lesions in two fish species. Based on these, the responses of each fish species against the pathogenic fungi were compared.

In the PBS-injected fish, very minimal pathological changes were seen. These were likely due to the damage caused by the insertion of the needle during inoculation procedures. Three-spot gourami and sand whiting injected with *A. invadans* spores developed mycotic granulomas and exhibited marked cellular infiltration, predominantly composed of macrophages and lymphocytes. According to Warren (1980), these are the usual reactions whenever there is damage or persistent foreign bodies in the tissues. The cells involved in chronic inflammation include the macrophages which transform into epithelioid cells, foreign-body giant cells, lymphocytes and plasma cells (Roberts 1989). Thus, even though the inflammation mechanism in fish is not yet fully understood, they show inflammatory reactions which are similar to those seen in mammals based on the type of leucocytes involved (Ellis 1986).

Macrophages play a very significant role in the pathogenesis of chronic inflammatory lesions since they are involved in phagocytosis and secretion of hydrolytic enzymes which constitute nonspecific immune responses in vertebrates. Lymphokines released by T lymphocytes were reported to attract macrophages to a particular site and to activate them to phagocytose (Davies and Allison 1976; Bainton 1980) and eventually degrade the infecting agent. This could explain the presence of PAS-positive cells, presumably macrophages which had engulfed hyphal debris, at day 8 until day 12 p.i.

In EUS lesions, the highly invasive *A. invadans* persists within fish tissues resulting in mycotic granulomas formed by layers of epithelioid cells around the penetrating fungal hyphae (Chinabut and Roberts 1999). This mechanism had been associated with various pathogens in fish (Balouet and Baudin-Laurencin 1986). Apparently, the capacity to ingest foreign materials is less in epithelioid cells than in mononuclear phagocytes. However, the secretory and the microbicidal capacity are increased when macrophages develop into epithelioid cells (Adams 1983).

Another histopathological characteristic observed in both fish species was extensive myonecrosis. This may possibly be due, in part at least, to proteolytic products from the fungi since isolates of *A. invadans* showed protease activity when grown in glucose-yeast agar supplemented with skim milk (Graeme Fraser, pers. comm.). It may also result from enzymes and reactive oxygen intermediates released by infiltrating macrophages (Adams 1983).

No giant cells were formed in either fish species. According to Ferguson (1989), multinucleated giant cells of the foreign-body type, have been seen only in experimental situations in fish. However, histopathological studies involving fish affected with mycotic granulomatosis showed that crucian carp, goldfish, ayu and the Japanese trident goby developed foreign-body giant cells (Miyazaki and Egusa 1972, 1973a, 1973b, 1973c; Miyazaki 1994) while bluegill, Formosan snakehead, black mullet (Miyazaki 1994) and dwarf gourami (Wada *et al.* 1994) did not exhibit any foreign-body giant cell formation. Thus, it is likely that this response is fish species-specific. In mammals, mature macrophages fuse to form giant cells which are short-lived *in vitro* with poor phagocytic activity probably due to reduced number and/or affinity of surface receptors (Warren 1980; Adams 1983). However, Secombes (1985) reported that multinucleate giant cells from the head kidney of rainbow trout, *Oncorhynchus mykiss*, showed some phagocytosis. Plasma cells were not particularly noted in the sections as the 8- to 12-day period was probably not sufficient for such cells to be formed.

Oedematous response was also observed in both species. In most cases of fish diseases with epidermal and dermal lesions, oedema is due to waterlogging, especially among freshwater species, when the osmotic gradient forces water into the underlying muscle tissues which results in severe necrosis and sloughing of the epidermis (Ferguson 1989). It is likely that the 3-spot gouramis were not able to cope with the breach in the osmotic barrier, thus the occurrence of higher mortality at days 7-8 p.i.

In 3-spot gouramis which did not develop external lesions, the pathological signs like thicker granulomas with fibrotic peripheries and necrotic centers were observed. Moreover, lytic activity was limited in the skeletal muscles. It is possible that as early as day 8 p.i., the healing process had started, as some sections showed newly regenerated muscle and granulation tissues, and thicker granulomas with fungal debris in the center. Wada (1996) reported that in carp injected with *Aphanomyces piscicida* zoospores the lesions were confined to smaller areas in the muscle because of the very intense inflammatory response against the pathogen. These signs possibly indicate that this species had a relatively effective defense against the fungal invasion, unless the fish die before the pathogen is removed. Since gouramis are laterally compressed compared with sand whiting which are torpedo-shaped, it is possible that the fungal hyphae could invade through a shorter distance from the muscle to the internal organs, disrupting vital metabolic processes which could lead to mortality. This suggestion could possibly explain the higher number of dead gouramis.

No difference were observed in the behavioural and clinical manifestations of the disease in these fish. Qualitative histopathology revealed some degree of difference in their response against the fungi. Quantitative data, however, showed marked differences in terms of granuloma formation and inflammatory cell infiltration.

The 3-spot gouramis exhibited a more aggressive response since as early as day 6 p.i., mycotic granuloma formation was quite extensive and the granulomas were present in almost the entire cross-section, reaching a peak response at day 8 p.i. These features were only observed at day 10 p.i. in sand whiting even though

granuloma formation was greater compared with the gouramis. The decline in the granuloma count in the 3-spot gourami at day 10 p.i. was due to the linking of granulomas by proliferating fibrous tissue. Formation of new blood vessels was also evident in 3-spot gourami at day 8 p.i. In sand whiting, granulomas increased until day 12 p.i. indicating that the fungus was still persistently invading the tissues. The same pattern was also evident in the infiltration of inflammatory cells. Peak response was at day 6 p.i. in 3-spot gourami, decreasing from day 8 to day 10 p.i. From day 2 to day 8 p.i., the gouramis exhibited greater cellular infiltration than sand whiting. Peak cellular infiltration in sand whiting was at day 10 p.i., but was still less than the maximum response shown by the gourami at day 6 p.i.

The two fish species used in these trials showed typical EUS lesions through intramuscular injections of *A. invadans* zoospores, hence, a model of the sequential pathology of the lesions was developed. Sand whiting are commonly affected by EUS outbreaks in estuarine areas in the eastern part of Australia, along with sea mullet and yellowfin bream (Pearce 1990; Fraser *et al.* 1992). Different species of gouramis (*Trichogaster pectoralis* and *Trichogaster trichopterus*) were also identified to be very susceptible to EUS as reported in several countries in Asia, such as Thailand, Philippines, Malaysia, Myanmar and Sri Lanka (Bondad-Reantaso *et al.* 1992; Lilley *et al.* 1992). Roberts *et al.* (1994) noted significant differences in host cellular response as some fish like snakehead and other air-breathing species usually continue to survive while other species die before chronic responses can be mounted. Similarly, differences in response were found in these experimental fish and probably reflects the different hosts' unique immune response to the pathogen.

Taking into considerations the different physiologies of the two test species, which meant that different culture conditions were required, it was not possible to make a clear-cut decision on the relative susceptibility of these fish to EUS. However, there were differences in responses to *A. invadans*, which could be summarised from the results presented in this Chapter, and thus permit comparison between the two species. Based on fish mortality, a higher number of three-spot gouramis died compared with the sand whiting. But the three-spot

gouramis which survived and were sampled at day 12 p.i. showed early resolution of lesions. Although the same behavioural and clinical manifestations were shown by the two test species, there was some degree of difference since external ulcerations were observed to appear earlier in three-spot gouramis (day 5 to day 8 p.i.) while in sand whiting, petechial hemorrhages were observed at day 5 p.i. and well-developed external ulcers were seen at day 10 to day 12 p.i. These clinical manifestations were corroborated by the early and more aggressive cellular response in three-spot gouramis than in sand whiting as shown by qualitative and quantitative histopathological analyses. Thus, the three-spot gouramis tended to have greater inflammatory and granulomatous response and the lesions healed more quickly.

Chapter Three*

Histopathology, including skin responses, of sand whiting, *Sillago ciliata* Cuvier, subjected to temperature changes and injected with, or exposed to, *Aphanomyces invadans* spores

***Experiment I forms part of a paper published in Fish Pathology,33:327-335.**

3.1 INTRODUCTION

EUS, a multifactorial disease, is now considered as one of the most important finfish health problems in the Asia-Pacific region (Roberts 1997). Although various pathogens have been associated with the disease, only the fungus *Aphanomyces invadans* can induce the typical chronic granulomatous response in susceptible fish (Lilley *et al.* 1998). However, it appears that initial skin damage is required before the fungus can infect fish. Callinan (1997) showed that acid-sulfate soil runoff could cause epidermal damage which predispose estuarine fish species to *A. invadans* invasion. Kanchanakhan *et al.* (1999) also reported that in Thailand, EUS-associated rhabdovirus could experimentally induce skin damage in snakehead that made the fish vulnerable to fungal infection.

In some Asian countries, EUS outbreaks occur seasonally, usually during the colder months. In the Philippines, low and/or fluctuating water temperatures coincide with the occurrence of the disease. Bondad-Reantaso *et al.* (1992) recorded a temperature range of 18.5 °C-24 °C during 1989-1990 outbreaks. Similar findings were obtained from the 1993-1995 trials conducted in experimental rice-fish plots in the Philippines, with low temperature as a consistent factor during EUS outbreaks (Lumanlan-Mayo *et al.* 1997; Callinan undated).

Chinabut *et al.* (1995) showed that *A. invadans*-infected snakehead exhibited minimal inflammatory response with extensive tissue damage when kept at 19 °C in contrast to infected fish held at 26 °C and 31 °C. Also, laboratory and field studies on "winter saprolegniasis" of channel catfish confirmed that a rapid drop in temperature induced immunosuppression which favoured *Saprolegnia* infection (Bly *et al.* 1993b). In addition, Quiniou *et al* (1998) reported that an abrupt decrease in temperature caused a decrease in the density of epidermal mucous cells in channel catfish challenged with *Saprolegnia* spores. They suggested that the subsequent mucus loss made the fish more susceptible to *Saprolegnia* infection.

In this chapter, two experiments were undertaken to determine the effects of gradual or rapid drop in water temperature in sand whiting. In the first experiment, the effects of temperature on the sequential histopathological changes in *A. invadans*-injected whiting were investigated.

Infection method and histopathological analyses employed were based on the model developed in the previous chapter. Sand whiting were specifically used in this experiment since the lesions they exhibit, as described in Chapter 2, were more characteristically consistent. Two groups of sand whiting were used: one group was fed with a formulated low fish oil diet while the other group was fed with a diet supplemented with fish oil. However, the effects of fish oil supplementation will be presented in Chapter Five which deals with prophylaxis.

In the second experiment, the effects of temperature on the epidermal thickness and skin mucous cells were investigated in sand whiting exposed to *A. invadans* spores. Different infection techniques were employed in order to explore and determine the role of decreasing water temperature in the pathogenesis of EUS. The effects of L-cysteine ethyl ester (L-CEE), a mucolytic agent, was also simultaneously undertaken in this experiment but these will also be presented in Chapter Five.

3.2 MATERIALS AND METHOD

3.2.1 Fish and husbandry conditions

Experiment I

Three hundred sixty (360) sand whiting fingerlings (6.8 ± 1.69 g) were distributed in 40-L glass tanks with undergravel filters and continuous aeration. Prior to acclimation in tanks, the fingerlings were divided into two: first group of sand whiting fingerlings was fed with a formulated low fish oil diet (Table 3.1) were used for this experiment while the second group was fed with a high fish oil diet. The fish were fed their respective diets for 42 days (6 weeks).

The sand whiting fingerlings (15 x 24 tanks) were acclimated at least for a week prior to any changes in water temperature and fungal spore injection. During acclimation, the fingerlings were fed daily with their respective diets at 2% body weight. One-third of the water in all tanks was replaced every two days. Seawater from Weymouth, north coast of Tasmania, diluted with municipal water and stocked in a 450-L plastic tank with continuous aeration was used for water exchange. Immersion heaters were used to set water temperature at the required range. During acclimation, salinity ranged from 8-10‰, alkalinity from 75-100 mgL^{-1} $CaCO^3$, NH_3-N from 0.1 to 0.5 mgL^{-1}, pH from 7.2-7.6, DO from 2.3-7.5 mgL^{-1} and temperature from 24.5 °C to 28.1 °C.

Table 3.1 Composition of the formulated low fish oil diet

Components		g/100 g feed
Crude protein	48.75 %	
Crude fats	4.50 %	
Fish meal		80.00
Bentonite		8.39
Dextrose		8.00
Carboxy-Methyl Cellulose (CMC)		2.00
Minerals		0.50
Vitamins		0.75
Stat-C		0.36

Experiment II

Sand whiting (9.9 ± 2.89 g mean weight) were acclimated for one week in 40 L glass tanks with undergravel filters and continuous aeration. Fish were fed to satiety with 1.5 mm Ø (diameter) dry salmon pellets. Partial water change was

done every two days using seawater diluted with dechlorinated tap water. Salinity, measured with an Iwaki® hand refractometer, was decreased from 7‰ to 1-2‰ prior to the addition of fungal mats in each tank. Daily temperature and DO levels, measured using an Oxy-guard® Handy Mk III meter, ranged from 25.7–26.2 °C and 5.7-6.3 mgL^{-1}, respectively. Ammonia-nitrogen (NH_3-N) was negligible (Aquasonic® kit) while pH ranged from 6.8-7.2 (Activon 209 pH meter).

3.2.2 Experimental design

Experiment I

Fingerlings fed with low fish oil diet (15 x 12 tanks) were divided into 4 treatment groups: a) negative (-) control, injected with PBS, no temperature manipulations; b) positive (+) control, injected with *A. invadans* zoospores, no temperature manipulations; c) sand whiting subjected to gradual temperature decrease and then injected with *A. invadans* zoospores and d) sand whiting subjected to rapid temperature drop and then injected with *A. invadans* zoospores. All treatments were done in 3 replicate tanks. For the gradual temperature drop, immersion heaters were adjusted to lower the acclimation temperature to at least 17 °C over a period of seven days. For the rapid temperature drop, the acclimation temperature was lowered to 17 °C over a period of 24 hours.

The water temperatures chosen for this experiment were based on data from an ACIAR-funded project on EUS in the Philippines obtained during the 1994 cold season (September-November) which showed that there was a gradual decrease of temperature over a two week period prior to detection of EUS lesions in sampled African catfish (*Clarias gariepinus*). Average daily temperature in the 6 experimental rice-fish plots was 25.7 °C, hence 26 °C was used as temperature for the control group. The lowest recorded temperature was 14.5 °C during the peak prevalence (80-95%) of EUS (Lumanlan-Mayo *et al.* 1997). In channel catfish, non-permissive temperature for T lymphocytes is 17 °C and 14 °C for B lymphocytes. Since the response to *A. invadans* infection is primarily cellular in nature, 17 °C was trialled as the minimum temperature in this experiment.

Experiment II

Based on the results of a preliminary trial (Appendix 3), the "net-shaking" stress technique to induce epidermal damage was not suitable for sand whiting, hence, for this trial, skin abrasion and cohabitation were used as methods to induce EUS which could be employed to determine the role of decreasing water temperature in *A. invadans* infection.

After acclimation in glass tanks (12 fish x 16 tanks), the fish were divided into two temperature regimes. Fish in 8 tanks were maintained at 26 °C while the other 8 tanks were subjected to gradual temperature decrease (acclimation temperature to 19 °C in 7 days) and then maintained at 19 °C during the experimental period.

For both temperature regimes, four treatments were used as follows: a) positive (+) control, with fungal mats being added every two days; b) skin abrasion and addition of fungal mats; c) cohabitation with fish previously injected with *A. invadans* spores and addition of fungal mats every two days, and d) diet supplementation with L-cysteine ethyl ester (L-CEE) and addition of fungal mats every two days. Each treatment was done in two replicate tanks.

Abrasion was performed on all appropriate groups at the time the water temperature reached 19 °C in the low temperature tanks. Fish were anesthetised with 60 ppm benzocaine and the blunt edge of a sterile scalpel blade was used to abrade the skin (1 cm^2) in the anterodorsal portion of each fish. Fish were then allowed to recover in aerated water and were then returned to their respective tanks.

Likewise in the cohabitation treatment, infected fish (4 fish per tank) were transferred to tanks with uninfected fish after the temperature had dropped to 19 °C.

3.2.3 Preparation of *A. invadans* fungal mats and zoospore suspension

Methods used to sporulate *A. invadans* and preparation of zoospore suspension were described in Chapter Two. A suspension of 1.5×10^3 spores mL^{-1} was used as inoculum for Experiment I. For the second experiment, 32 fungal mats were produced in glucose-yeast broth as in Chapter Two, washed 5 times in autoclaved distilled water, placed in plastic histocassettes (2 mats x 16 histocassettes) and distributed in the experimental tanks (one histocassette per tank). This was done every two days during the experimental period.

3.2.4 Fish inoculation with *A. invadans* spores and addition of fungal mats in tanks.

After the acclimation period in Experiment I, fish were anesthetised with 50-60 ppm benzocaine and injected intramuscularly with 0.05 ml of 1.5×10^3 spores mL^{-1} suspension. After inoculation, fish were revived in well-aerated water and returned immediately in their respective tanks. Control fish were injected with 0.05 ml PBS.

For experiment II, the addition of fungal mats commenced after the acclimation period for both temperature treatment groups. This was to ensure that *A. invadans* zoospores were already present in the tanks once the temperature reached 19 °C. This was done continuously every two days until the experiment was terminated.

3.2.5 Sampling and experimental conditions

Experiment I

Fish previously subjected to temperature manipulations were maintained at 17 °C inside a refrigerated room after inoculation. Daily feeding was resumed a day after inoculation at 2% body weight for the control fish and 1.0% body weight for the temperature-challenged fish. Water quality variables were monitored during the experiment. One-third to one-half of the water was replaced every two days

for each tank. Mortalities and gross signs of fungal infection were recorded daily. One fish from each treatment tank was euthanased in 200 ppm benzocaine at 24-hr, 2, 4, 6, 8, 10, 12, 14, 16, and 18 days post-inoculation (p.i.) and samples were fixed for histopathology.

Experiment II

Fish which were subjected to a gradual temperature drop were maintained at 19 °C inside a refrigerated room during the experiment. Fish were continuously fed with Gibson's commercial dry salmon pellet (1.5 mm Ø), but less feed was given to fish held at low temperature. Water quality and fish mortality were monitored during the experiment and partial water change was done twice a week.

Samples for histopathology were collected at days 1, 3, 6, 9 and 12 of the experiment. Fish were sacrificed by hitting their head with the wooden handle of a laboratory spatula and were transversely cut in half. The anterior sections (with the head) were immediately fixed in 2.5% cacodylate-buffered glutaraldehyde with 2% Alcian blue to stain the mucus coat (Powell *et al.* 1992).

3.2.6 Histopathology

In Experiment I, cross-sections of fish fixed in 10% neutral buffered formalin were trimmed, decalcified, processed, embedded in paraffin wax, sectioned at 4-5 μm and stained using the periodic acid-Schiff (PAS) technique. Sections were qualitatively analysed for histopathological changes using an Olympus BH-2 light microscope. Quantitative analyses, which included mycotic granuloma counts and area measurement of cellular infiltration, were also done as described in Chapter Two.

In Experiment II, the samples were fixed for 24 hours and a small portion of the dorsal muscle, just anterior to the dorsal fin was carefully sliced with a sharp, single-edge blade and coated with 10% gelatine. The coated samples were further fixed for another 24 hours. The tissue samples were then washed with cacodylate buffer and transferred to 70% ethyl alcohol. The JB4 resin embedding kit (Agar

Scientific Ltd., England) was used instead of paraffin. Prior to embedding, the samples were transferred to 95% ethyl alcohol, left overnight in a shaker for infiltration. Sections were embedded in Beem embedding capsules with resin mixture prepared as described in the embedding kit instructions. The resin polymerised after 30-45 min and samples were trimmed prior to sectioning. Sections (4-5 μm) were cut using glass knives. Sections were stained with periodic acid-Schiff (PAS) and hematoxylin-eosin (H&E).

Using an Olympus BH-2 light microscope with a micrometer eyepiece, 10 random measurements of the epidermal thickness, from the basement membrane to the surface of the epidermis, were taken from one scale unit of each sample at x400 magnification. Mucous cells were counted in 250 μm length of epidermis in one scale unit per sample. No measurements were taken from the abraded fish. Moribund and some dead fish were collected and processed for routine histopathology. Paraffin sections of abraded fish and infected fish used in the cohabitation treatments were also prepared. These were stained with hematoxylin-eosin (H & E) and periodic acid-Schiff's (PAS) reagent, counterstained with Gill's hematoxylin.

3.2.7 Statistical analysis

Experiment I

Quantitative data were analysed using JMP version 3.1 statistical program. For the baseline temperature effects, data from the low-fish oil treatment group were used. Mycotic granuloma counts were transformed using the square root transformation while percentage cellular infiltration data were arcsine square root transformed. Data were tested for homogeneity of variance prior to two-way analysis of variance (ANOVA, full factorial) with temperature and sampling day and their interaction as factors. These were followed by Tukey-Kramer HSD tests to detect difference between treatment means.

Experiment II

Epidermal thickness measurements and mucous cell counts from day 1 to day 9 were statistically analysed since most fish in the two treatments [(+) control and cohabitation] had died at day 12. Full factorial 3-way ANOVA was used with temperature, infection method and sampling and their interactions as factors. Data were subjected to normality and homogeneity of variance tests prior to the ANOVA and were arcsine square root-transformed. Multiple comparison of treatment means was done using Tukey-Kramer HSD test.

3.3 RESULTS

3.3.1 Experiment I

3.3.1.1 Water quality during the experiment

Table 3.2 shows the range of values for the different water quality variables measured during the experimental period.

Table 3.2 Range of values for each water quality variable measured during the experiment.

	Salinity (‰)	Dissolved Oxygen (mg/l)	Temperature (°C)	NH_3-N (mgL^{-1})	pH	Alkalinity (mgL^{-1} $CaCO_3$)
Low Fish Oil Diet						
1. (-) Control	7-12	5.4-8.1	25.5-26.6	<0.1	7.2	75
2. (+) Control	7-11	4.6-7.9	25.1-26.7	<0.1-0.5	7.2-7.4	75
3. Gradual drop	7-12	6.8-8.6	17.2-17.4	<0.1-0.5	7.2-7.4	75
4. Rapid drop	7-11	6.7-8.8	17.1-17.5	<0.1	7.2	75

3.3.1.2 Fish mortality and gross observations

Fish mortality (Table 3.3) during the experiment was minimal in the different treatment tanks. Mortality in the (-) control fish could be attributed to handling stress during the injection of PBS. Some fish jumped out of the tanks and were deleted from the trial.

Table 3.3 Sand whiting mortality in Experiment I.

Low fish oil	(-) Control	(+) Control	Gradual drop	Rapid drop
Tank 1	0/15	1/15	0/14	0/15
Tank 2	0/15	1/15	0/13	0/15
Tank 3	2/15	1/13	0/14	0/14

At day 2 p.i., some fish in the (+) control group had pale and inflamed injection sites and had lost scales over the injection sites at day 5 p.i. While (-) control fish ate and swam actively, most of the fish injected with fungal spores were lethargic during the experiment. Fish held at low temperature had reduced appetite. At day 11 p.i., most (+) control fish had external lesions and hemorrhages and, in particular, while fish held at low temperature had highly inflamed injection sites.

3.3.1.3 Histopathology

Qualitative examination

PBS-injected fish. At 24 hr p.i., the fish showed very minimal cellular infiltrate streaming along a myoseptum with some degenerating muscle fibres. At day 2 p.i., degeneration and lysis of muscle fibres was greater than at 24 hr p.i. but the sarcoplasmic membranes and nuclei were still present. Inflammatory cells, predominantly macrophages were streaming along myosepta. The skin, including

the scales, remained unaffected. At day 4 to day 6 p.i., the fish exhibited regenerating muscle fibres and very minimal cellular infiltrate. At day 8 p.i., the muscle fibres had fully regenerated.

Zoospore-injected fish. The sand whiting held at high temperature exhibited similar histopathological changes as described in the previous chapter. Fish subjected to gradual or rapid temperature drop showed very similar pathological responses to *A. invadans* injection. These fish showed very slow response to the injection, as compared with those maintained at higher temperature. Very minimal cellular infiltration and delayed granuloma formation were observed. Extensive myonecrosis was evident and onset of reparative processes, like fibroplasia, were likewise delayed.

Table 3.4 shows the comparison between fish maintained at high temperature and fish subjected to either gradual or rapid drop in temperature. Figs. 3.1, 3.2, 3.3, 3.4 and 3.5 further demonstrate the qualitative changes exhibited by both groups of fish.

Quantitative analyses

Mycotic granuloma counts (Fig. 3.6)

Statistical analysis showed there was significant difference between treatments due to temperature (P= <0.001), sampling days (P=<0.001) and interaction of temperature and sampling days (P=<0.001). During the experimental period, significantly higher granuloma counts were detected at days 14 and 16 p.i. in injected fish held at high temperature. For fish held at low temperature, highest granuloma count was at day 18 p.i. which was not significantly different to the granuloma count at day 10 p.i. of fish held at high temperature. Fish which were subjected to either rapid or gradual drop in temperature showed noticeable mycotic granulomas only at day 10 p.i. while fish held at high temperature had distinct granulomas at day 4 p.i. This shows a delay of 6-8 days in the granulomatous response of the fish kept at 17 °C.

Table 3.4 Comparison of pathological changes in fish injected with *A. invadans* spores subjected to high or decreasing water temperature.

Days post-injection	High temperature + spores	Decreased temperature + spores
1	Cellular infiltrate running along myosepta, muscle lysis scattered/ involving several muscle bundles.	Very minimal cellular infiltration, muscle lysis usually involving one muscle bundle.
2	Cellular infiltrate reaching layer between muscle and *stratum compactum.*	Same as above.
4	Cellular infiltrate spreading to contralateral muscle bundles, fungal hyphae enveloped within epithelioid cells, skin not affected.	No fungal granulomas, limited inflammatory cells; scattered muscle lysis.
6	Focal dermatitis, both sides of the epaxial muscles with cellular infiltrate and myonecrosis evident, thicker fungal granulomas.	Inflammatory cells present in the contralateral muscle and some parts of the dermis, fungal hyphae present but no granulomas, greater degree of tissue damage.
8	Dermis with fungal granulomas, muscle necrosis and inflammatory cells still spreading, fibrous tissue starts to develop around granulomas, kidney with fungal granulomas.	Dermatitis, fungal hyphae scattered in the muscle area, onset of granuloma formation, myonecrosis and inflammatory cells still spreading.
10	Epidermis inflamed with some areas eroded, necrotising dermatitis, cellular infiltrate in the hypaxial muscles, granulomas starting to mature.	Some areas of the s. compactum inflamed, some fungal granulomas present (1-2 cell layer thick).
12	Epidermis lost, necrotising dermatitis, granulomas enlarged/linked together due to fibrosis, lost muscle replaced by granulomas and free macrophages and lymphocytes, lysis of hypaxial muscles.	Localised necrotising dermatitis, fungal granulomas 3-4 cell layer thick but much less in number; most fungal hyphae not enclosed within epithelioid cells.
14	Extensive fibrosis, most granulomas linked, and with cellular debris in the centre..	Some parts of the epidermis eroded, necrotising dermatitis with fungal granulomas, thicker and linked granulomas in the muscle area, onset of fibrosis.
16	Almost entire section affected, fungal debris within mature granulomas, peripheral epidermis hyperplastic	Same as in day 14 p.i.
18	Granulomas with fungal debris (outer fibrous envelop still present) in the hollowed centre, some regenerating muscle fibres present.	Epidermis either lost or eroded, lesion concentrated to both sides of the epaxial muscles but some inflammatory infiltrate had spread in the hypaxial muscles.

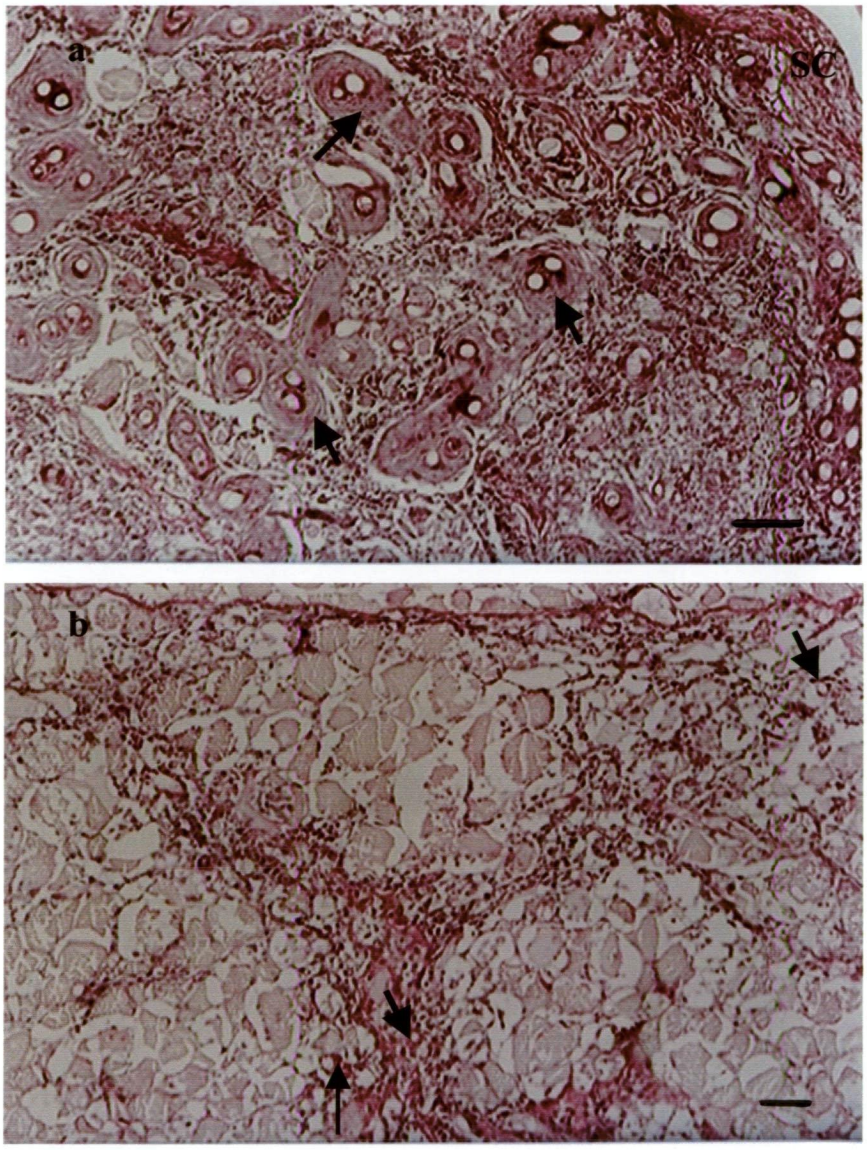


Figure 3.1 Photomicrographs of sand whiting at day 8 p.i. **a) high temp**: necrotic muscle fibres replaced by extensive mycotic granulomas (arrows) and cellular infiltration in the affected area; stratum compactum (SC) was also highly inflamed. **b) gradual temp drop**: few fungal hyphae (arrows) visible but no granulomas, limited cellular infiltrate but muscle necrosis was evident. (PAS stain, bar= 100 μm)

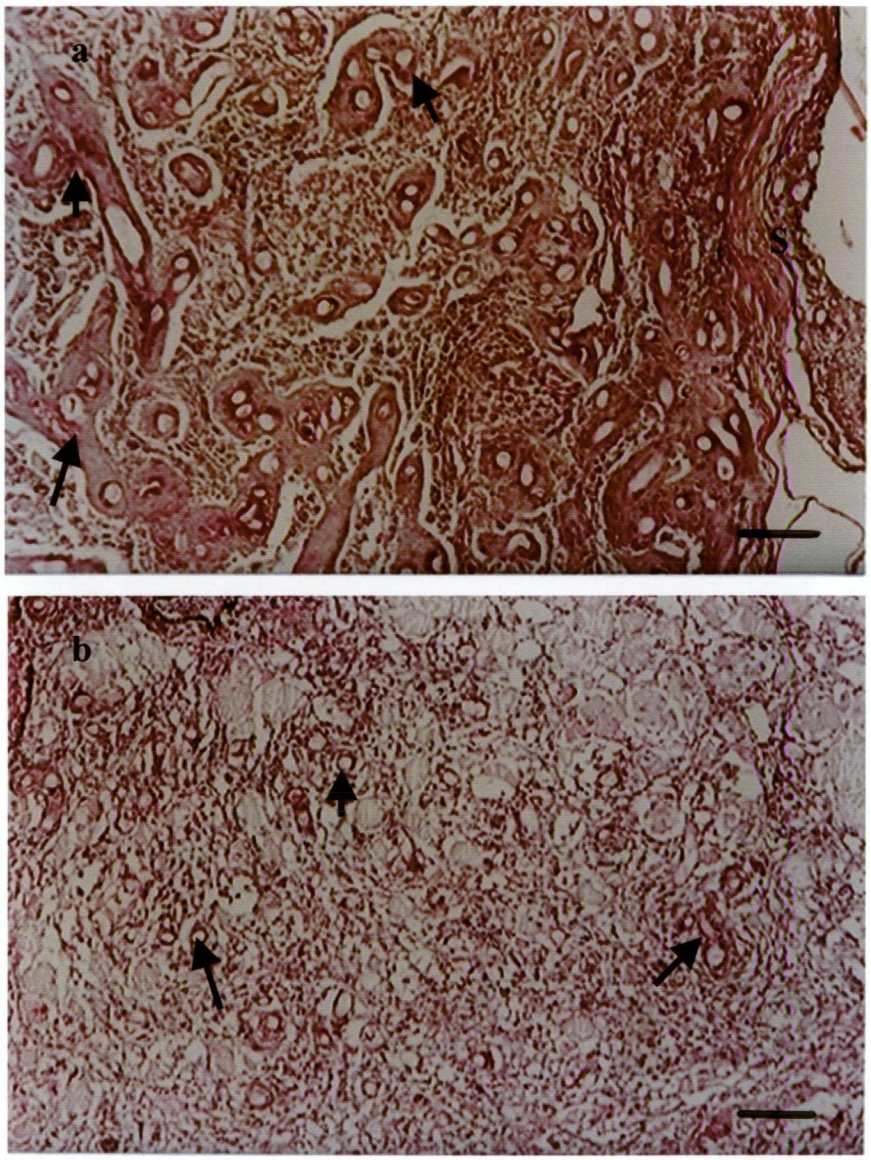


Figure 3.2 Photomicrographs of sand whiting at day 10 p.i. **a) high temp**: skin (S) highly inflamed and necrotic with fungal granulomas while granulomas (arrows) in the muscle area were more fibrous and linked together. **b) rapid temperature drop**: fungal hyphae(arrows) present with some degree of granuloma formation, tissue damage was extensive. (PAS stain, bar= 100 μm)

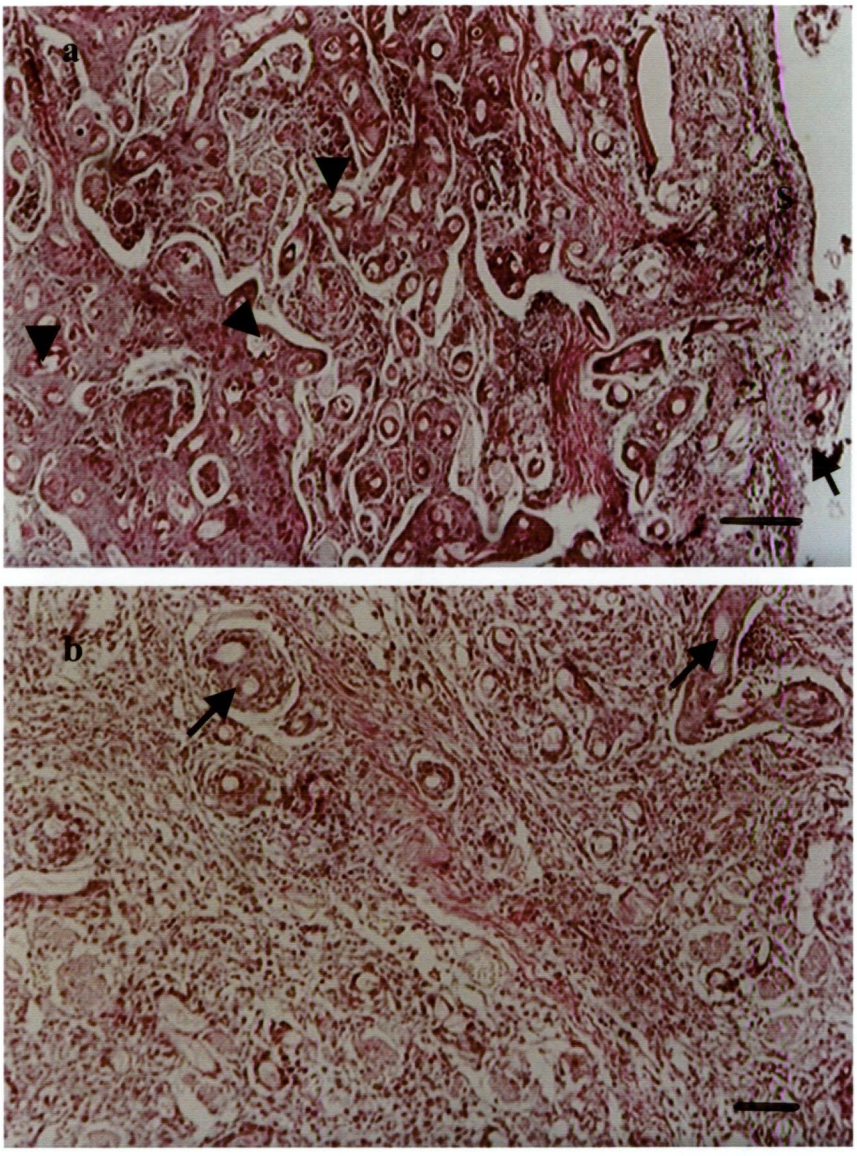


Figure 3.3 Photomicrographs of sand whiting at day 14 p.i. **a) high temp.**: most granulomas were mature and linked together with some debris (arrowheads) in the centre; skin (S) highly inflamed and eroded (arrow) in some areas. **b) gradual temperature drop**: onset of fibrosis with some granulomas linked together (arrows), extensive cellular infiltration. (PAS stain, bar= 100 μm)

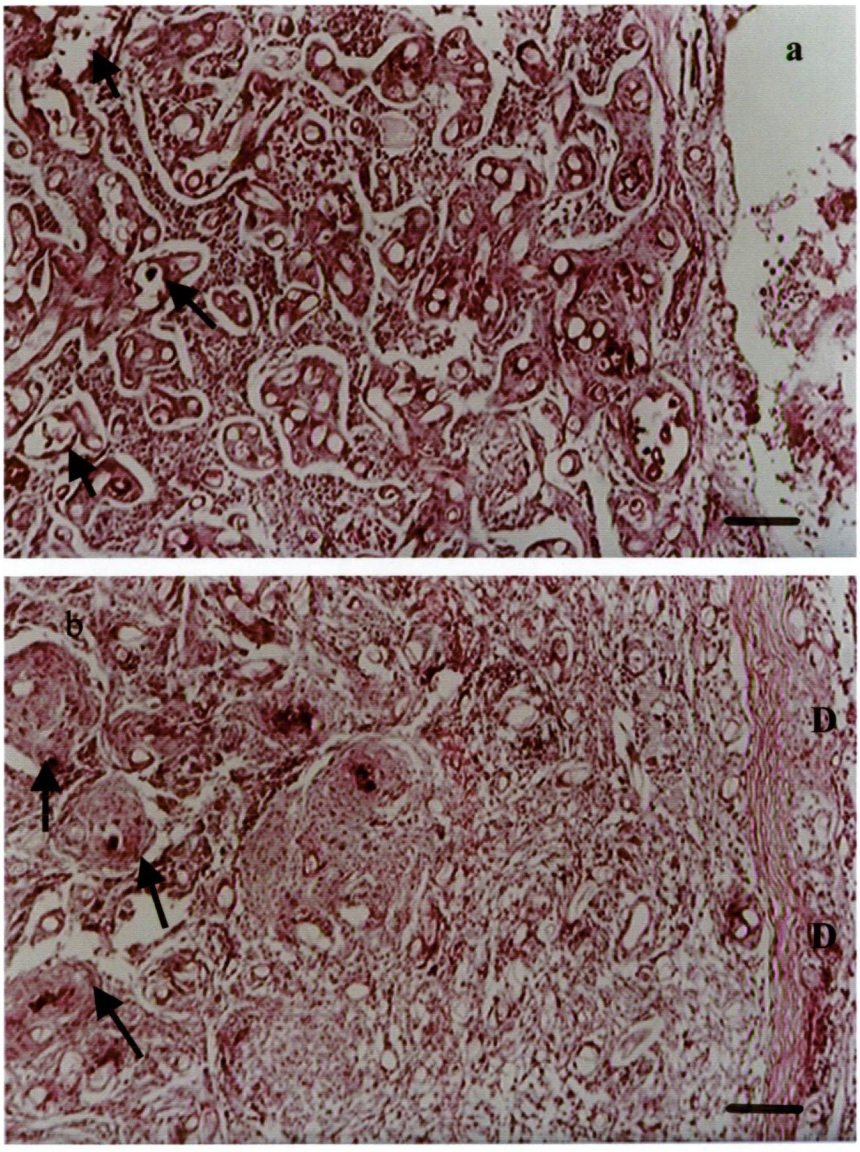


Figure 3.4 Photomicrographs of sand whiting at day 16 p.i. **a**) high temp: epidermis sloughed off, fungal debris within necrotic granulomas (arrows). **b) rapid temperature drop**: dermatitis (D) with fungal invasion, thicker and highly fibrous mycotic granulomas (arrows) and oedema in the muscle area. (PAS stain, bar= 100 μm)

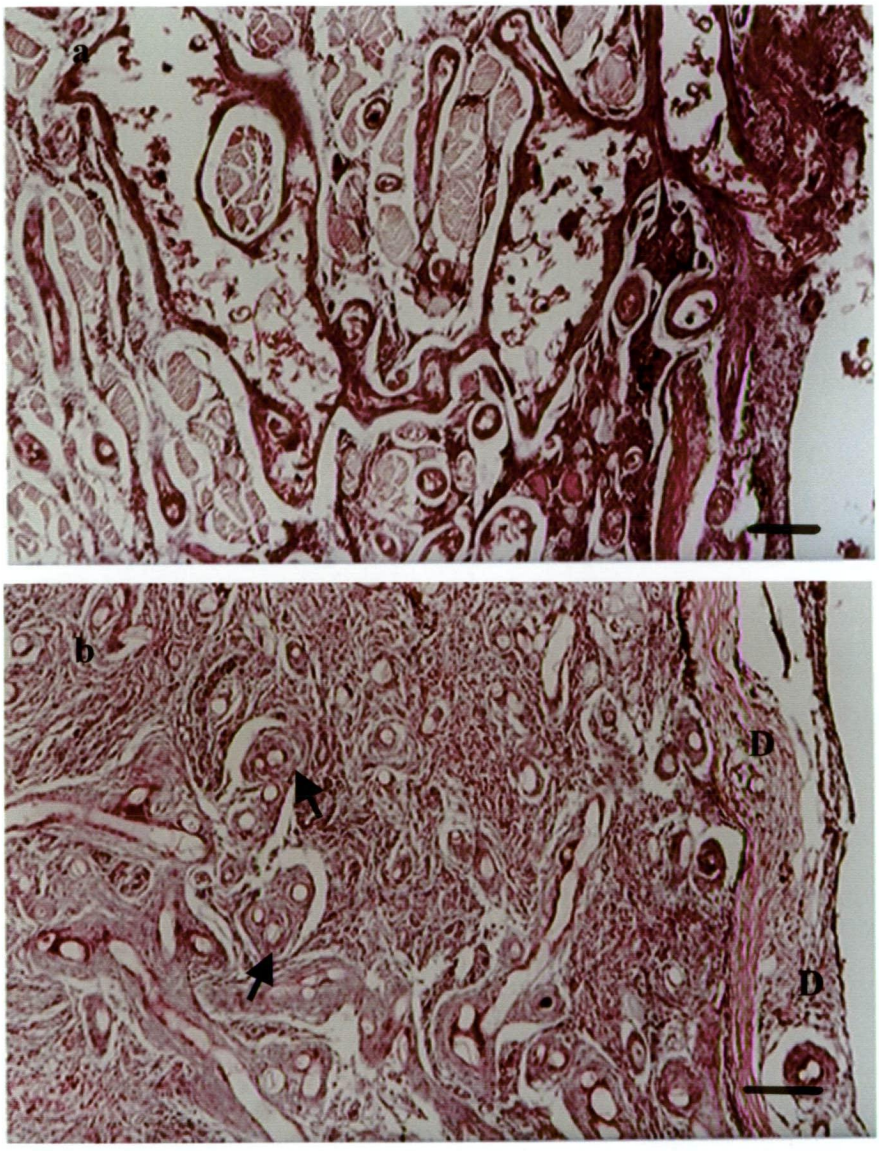


Figure 3.5 Photomicrographs of sand whiting at 18 day p.i. **a) high temp**: necrotic granulomas (with remnant fibrous periphery) with fungal debris or with regenerating muscle fibres in the centre; **b) rapid temperature drop**: dermatitis (D) with distinct mycotic granulomas, linked granulomas (arrows) due to fibrosis in the muscle area. (PAS stain, bar= 100 μm)

At all sampling periods (Table 3.5), the mean granuloma counts of the fish held at high temperature were significantly higher than those at low temperature treatments, except at day 18 p.i. when there was no significant difference among the treatments. At this stage, fish held at high temperature had lysed the fungi and regeneration of muscle fibres had started.

Percent cellular infiltration (Fig. 3.7)

Two-way ANOVA also showed significant differences between treatments due to temperature (P=<0.001), sampling day (P=<0.001) and interaction of temperature and sampling day (P=<0.001). Significantly higher percentage of cellular infiltration was detected at day 14 p.i. and at day 16 p.i. for fish held at high temperature but at day 18 p.i., this was not significantly different from that found in fish held at low temperature. Generally, fish held at high temperature had greater inflammatory cell response than the fish held at low temperature during the experiment (Table 3.6).

3.3.2 Experiment II

3.3.2.1 Water quality during the experiment

During the experiment, temperature ranged from 25.5 °C to 26.2 °C while DO level was from 6.4 mgL^{-1} to 7.2 mgL^{-1}. In the low temperature set-up, temperature ranged from 19.0 °C to 19.2 °C and DO level was from 7.5 mgL^{-1} to 8.2 mgL^{-1}. Salinity was from 0-2‰; NH_3-N was <0.25 mgL-1; NO_2^- from <0.1-0.2 mgL^{-1} and pH ranged from 5.4-6.5.

3.3.2.2 Fish mortality

Table 3.7 shows the fish mortality during the three phases of the trial: acclimation, temperature manipulation and main experimental period. As in the

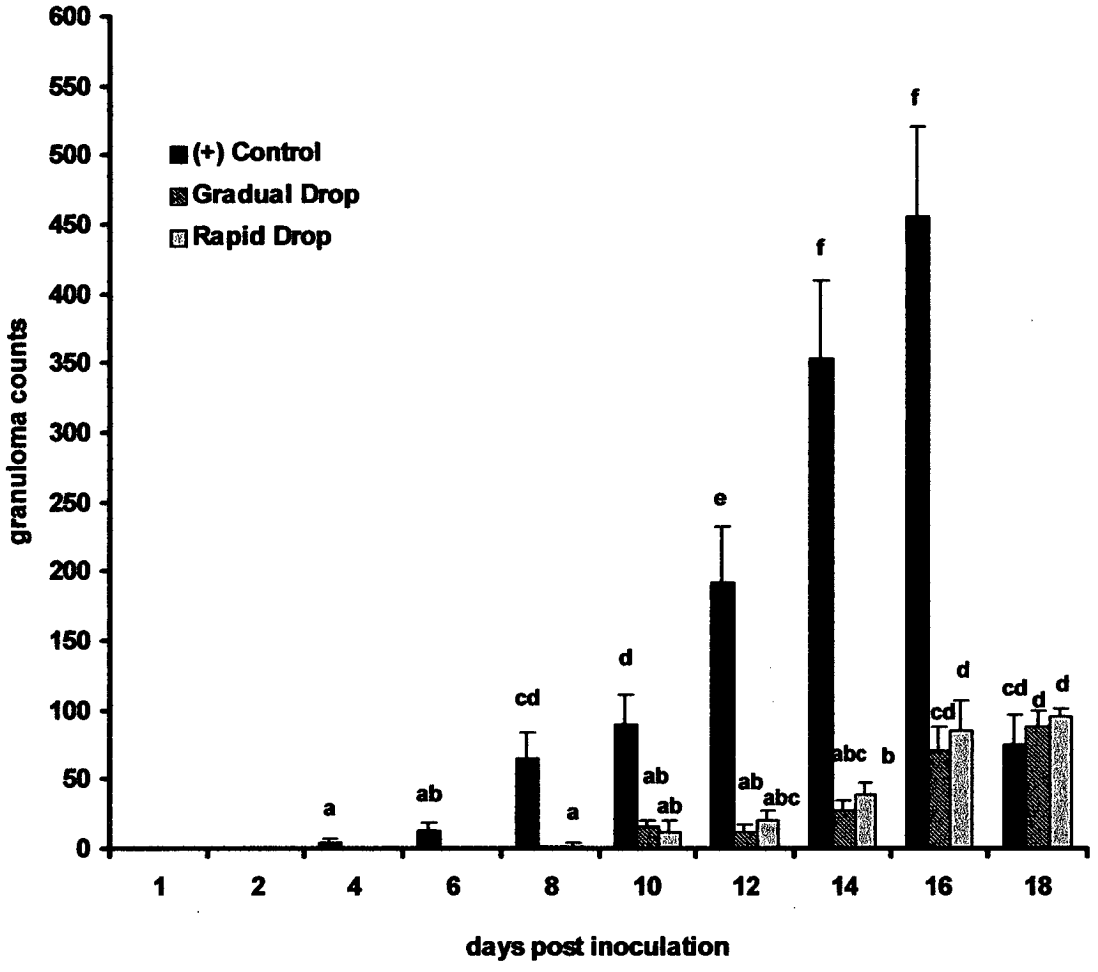


Figure 3.6 Mycotic granuloma counts in *A. invadans*-infected sand whiting subjected to temperature variations. Bars are means ± s.e. of three replicates; common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

Table 3.5 Mycotic granuloma counts in *A. invadans*-injected sand whiting. Values are means ± s.e. of three replicates; means followed by a common letter within a row are not significantly different by Tukey-Kramer HSD test at $P<0.05$.

Days Post Infection	(+) Control	Gradual Temp Drop	Rapid Temp Drop
1	0	0	0
2	0	0	0
4	5.0 ± 2.5^{a}	0^{a}	0^{a}
6	13.7 ± 4.4^{a}	0^{b}	0^{b}
8	65.3 ± 18.3^{a}	2.0 ± 0^{b}	2.0 ± 2.0^{b}
10	90.0 ± 21.7^{a}	15.7 ± 4.9^{b}	11.7 ± 8.0^{b}
12	191.3 ± 40.3^{a}	11.3 ± 6.6^{b}	20.7 ± 6.4^{b}
14	353.3 ± 56.2^{a}	26.7 ± 7.7^{b}	39.3 ± 9.0^{b}
16	456.0 ± 64.6^{a}	70.3 ± 17.6^{b}	84.7 ± 22.3^{b}
18	75.0 ± 15.0^{a}	88.7 ± 10.7^{a}	94.7 ± 5.8^{a}

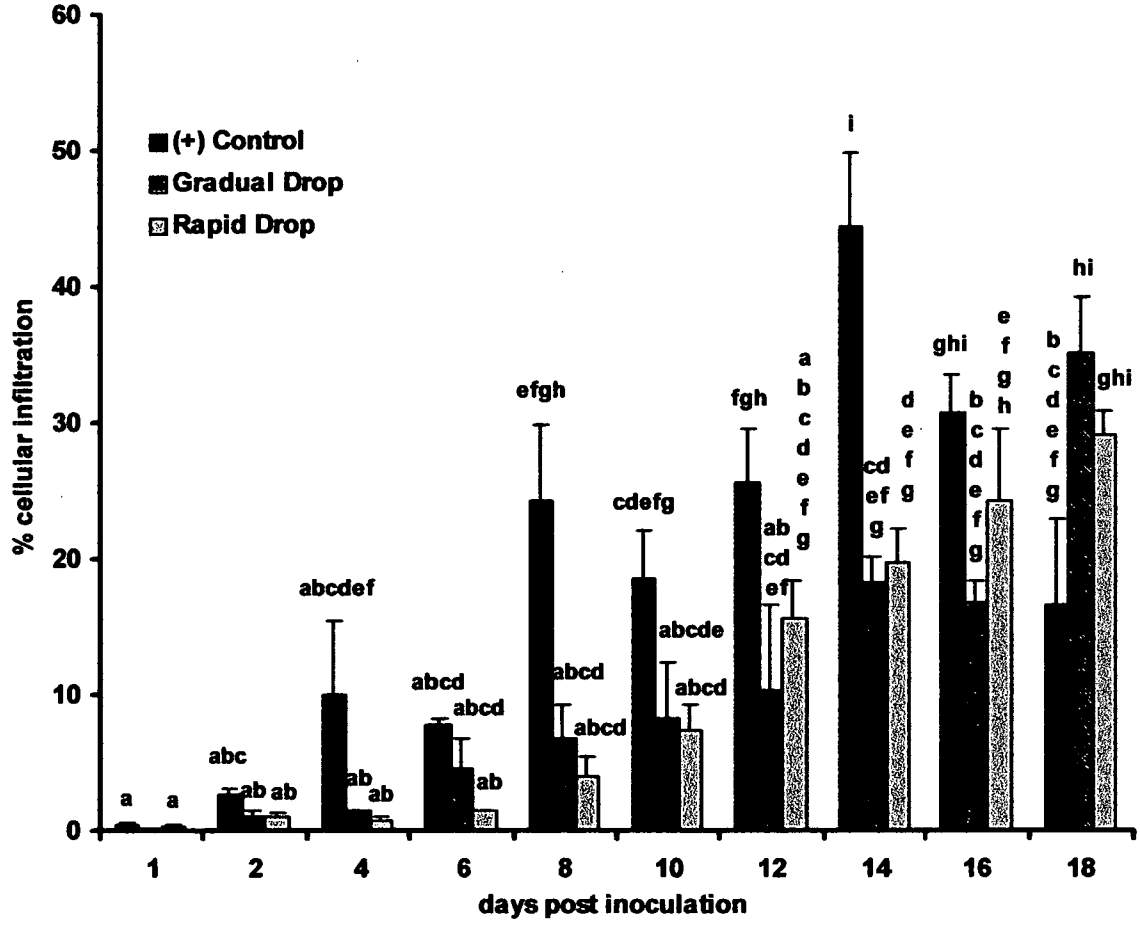


Figure 3.7 Percentage cellular infiltration in *A. invadans*-infected sand whiting subjected to temperature variations. Bars are means ± s.e. of three replicates; common letters not significantly different byTukey-Kramer HSD test at P=<0.05.

Table 3.6 Percentage cellular infiltration in *A. invadans*-injected sand whiting. Values are means ± s.e. of three replicates; means followed by a common letter within a row are not significantly different by Tukey-Kramer HSD test at P=<0.05.

Days Post Infection	(+) Control	Gradual Temp Drop	Rapid Temp Drop
1	0.5 ± 0.12[a]	0.1 ± 0.07[a]	0.3 ± 0.12a
2	2.6 ± 0.47[a]	1.0 ± 0.51[a]	1.0 ± 0.26a
4	10.0 ± 5.37[a]	1.4 ± 0.10[a]	0.7 ± 0.26
6	7.8 ± 0.43[a]	4.6 ± 2.25[ab]	1.4 ± 0.14b
8	24.2 ± 5.73[a]	6.7 ± 2.59[b]	4.0 ± 1.35b
10	18.5 ± 3.48[a]	8.2 ± 4.19[a]	7.4 ± 1.79a
12	25.6 ± 3.90[a]	10.3 ± 6.29[a]	15.6 ± 2.78a
14	44.4 ± 5.49[a]	18.2 ± 1.88[b]	19.7 ± 2.49b
16	30.7 ± 2.78[a]	16.7 ± 1.66[b]	24.2 ± 5.36ab
18	16.6 ± 6.44[a]	35.1 ± 4.20[a]	29.1 ± 1.86a

preliminary trial (Appendix 3), there was heavy mortality in the different treatments. Prior to the main experimental period, only 2 fish were left in one tank which was designated for a cohabitation and fungal mats treatment at 26 °C, hence there was no replicate tank for this treatment. Another tank of fish which were fed with L-CEE-supplemented diet also died when the temperature was gradually dropped from 26 °C to 19 °C. During the main experimental period, more sand whiting died in the treatments held at low temperature compared to the fish kept at 26 °C. Almost all of the abraded fish held at 19 °C had died after day 6 while for the (+) control all fish had died after 8 days.

3.3.2.3 Gross observations and histopathology

Most of the dead and moribund fish during the experiment had pinpoint hemorrhages on the dorsum immediately below the dorsal fin. Histopathological examination showed erosion of the epidermis, with or without associated bacteria and fungal elements. However, no gross or histopathological signs typical of EUS lesions were observed. For the abraded fish kept at 26 °C, the scarified area was inflamed but bacterial or fungal presence near the area was only seen in 1/7 fish. However, in fish held at 19 °C, 7/13 fish examined showed bacteria and/or fungal elements near the abraded area (Fig. 3.8a). The *A. invadans*-injected fish used in the cohabitation treatment were confirmed to be EUS-infected but were unable to induce infection in other experimental fish (Fig. 3.8b). The involvement of fungal elements was first observed in the sample obtained on the 4th day after the first fungal mats were added in the experimental tanks.

Fig. 3.9a shows the normal skin structure of sand whiting. The epidermis is composed of 8-10 layers of epithelial cells with mucous cells interspersed conspicuously within the cell layers. The dermis is made up of the *stratum spongiosum* and *stratum compactum* as in other finfish. In this experiment, some fish exhibited vacuolated epithelial cells in the epidermis (Fig. 3.9b), especially the cells near the surface. This was predominantly observed in abraded fish but some fish from the other treatments for both temperature levels also showed epidermal vacuolation and oedema. Necrotic or degenerating epidermal areas were also seen in some sections.

Statistical analysis of epidermal thickness measurements and mucous cell counts taken from a scale unit (Fig. 3.10) did not show any significant difference between treatments [(+) control and cohabitation] used in the experiment. There was high variation in the epidermal thickness data within treatments. The mucous cell counts were generally low in the three treatments. Fish which exhibited epidermal spongiosis and vacuolations generally lacked mucous cells. Table 3.8 shows the epidermal thickness data and the mucous cell counts for this trial.

3.4 DISCUSSION

In the first experiment, qualitative and quantitative histopathological analyses showed that fish subjected to either rapid or gradual drop in temperature, and subsequently maintained at low temperature (~17 °C), exhibited delayed and reduced capacity to form granulomas in response to *A. invadans* spore injection. The influx of inflammatory cells was also reduced. The onset of muscle regeneration and other reparative processes were likewise delayed. Thus, the results from this study confirmed the findings of Chinabut *et al.* (1995) with *A. invadans*-injected snakehead held at different temperatures. In their fish, histopathology revealed marked reduction in leukocytic infiltration with severe myonecrosis in fish held at 19 °C. There was also high mortality in snakehead maintained at low temperature. However, in my experiment, mortality associated with the fungal infection was very low in sand whiting, even in fish held at 17 °C. It is likely that snakehead are relatively more susceptible to *A. invadans* than sand whiting and it is also possible that the amount of zoospores inoculated was higher compared with the inoculum used in this study, but this cannot be confirmed from their paper.

As a component of a chronic inflammatory response, granuloma formation gives a degree of protection by containing the pathogen within the infected area. Without this mechanism, the host will experience a prolonged exposure to pathogens that could lead to severe tissue damage. This can explain the marked myonecrosis during fungal hyphae invasion observed in sand whiting held at low temperature.

Table 3.7 Sand whiting mortality in Experiment II.

Tank no.*	Mortality during acclimation (one week)	Temperature treatments	Mortality during week 1 of trial	Experimental treatments	Mortality during main experiment (12 days)
1	0/12	26 °C	2/12	Fungal mats only	0/10
2	2/12	26 °C	5/10	Cohabitation + mats	0/5
3	1/12	26 °C	1/11	Fungal mats only	2/10
5	1/12	26 °C	1/11	Abrasion + mats	2/10
6	1/12	26 °C	9/11	Cohabitation + mats	2/2
8	2/12	26 °C	2/10	Abrasion + mats	2/8
10	1/12	26-19°C	1/11	Cohabitation + mats	5/10
11	1/12	26-19 °C	2/11	Abrasion + mats	7/9
12	2/12	26-19 °C	3/10	Cohabitation + mats	5/7
13	5/12	26-19 °C	2/7	Fungal mats only	1/5
14	2/12	26-19 °C	2/10	Fungal mats only	4/8
16	0/12	26-19 °C	0/12	Abrasion + mats	9/12

*Tanks 4, 7, 9 and 15 were L-CEE + mats treatments.

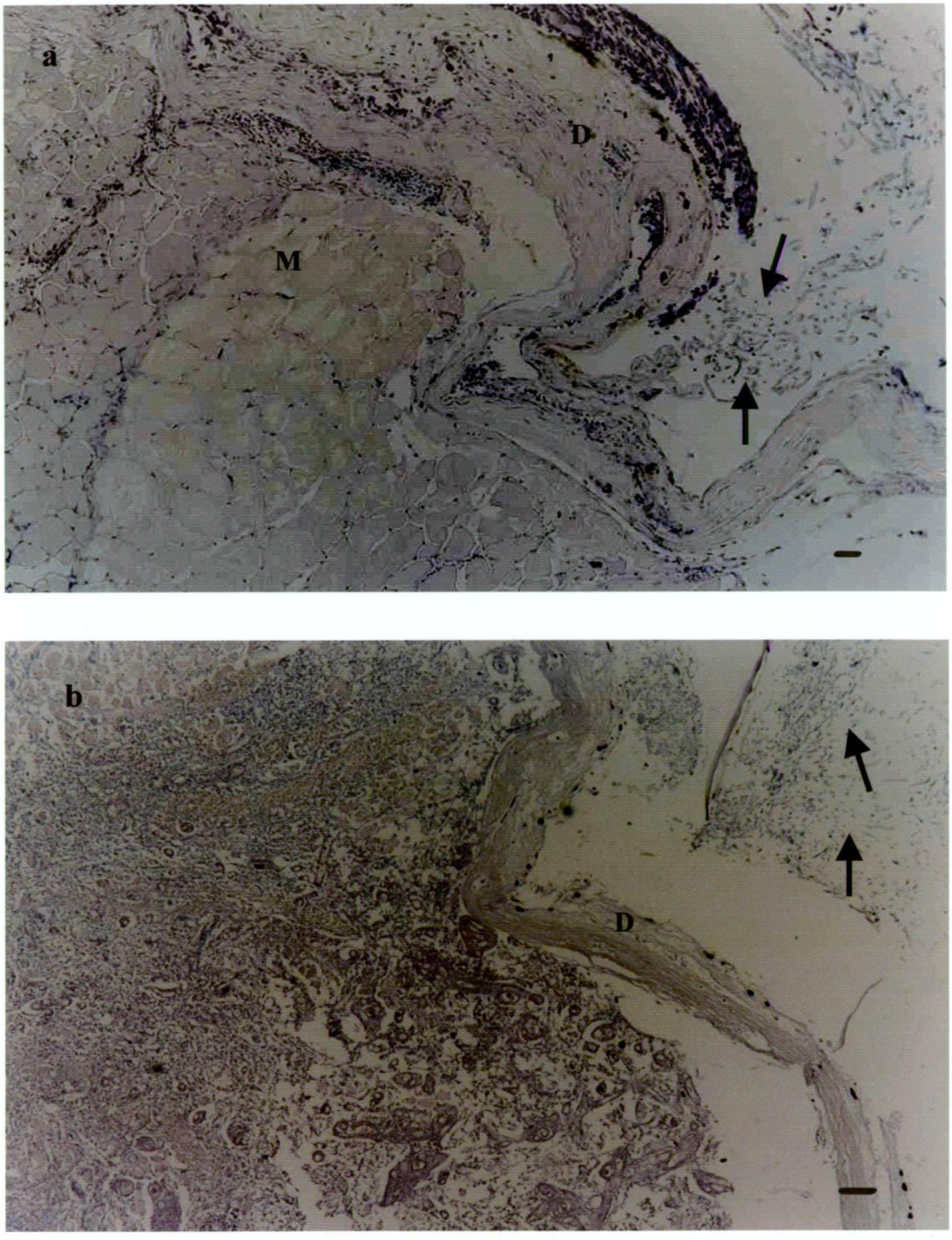


Figure 3.8 a) Sand whiting with abraded skin held at 19 ℃ exhibited minimal cellular infiltration in the dermis (D) and muscle (M) area; fungal hyphae were present (arrows) near the scarified skin (PAS, bar=100 μm). **b)** Sand whiting held at 26 ℃ and injected with *A. invadans* spores used in the cohabitation treatment; fungal hyphae (arrows) were also present near the necrotising dermis (D). (PAS stain, bar=150 μm).

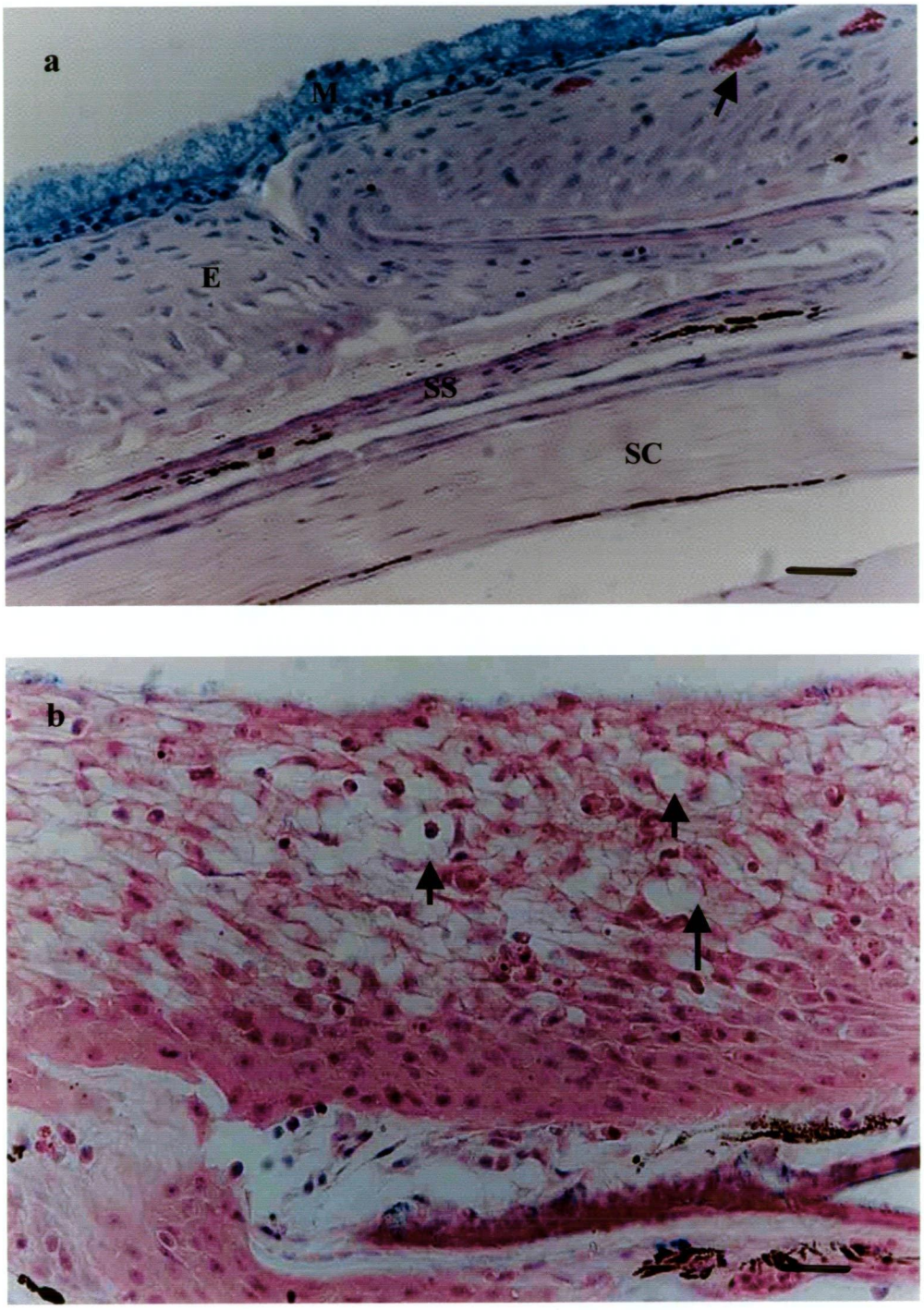


Figure 3.9 a) Normal skin structure of sand whiting: E-epidermis; mucous cell (arrow); M-mucus; SS-*stratum spongiosum*; SC-*stratum compactum* (PAS, resin section). **b)** Sand whiting exposed to *A. invadans* only and kept at 26 ºC showed vacuolated (arrows) and thicker epidermis (H/E, resin section). bars=25 µm.

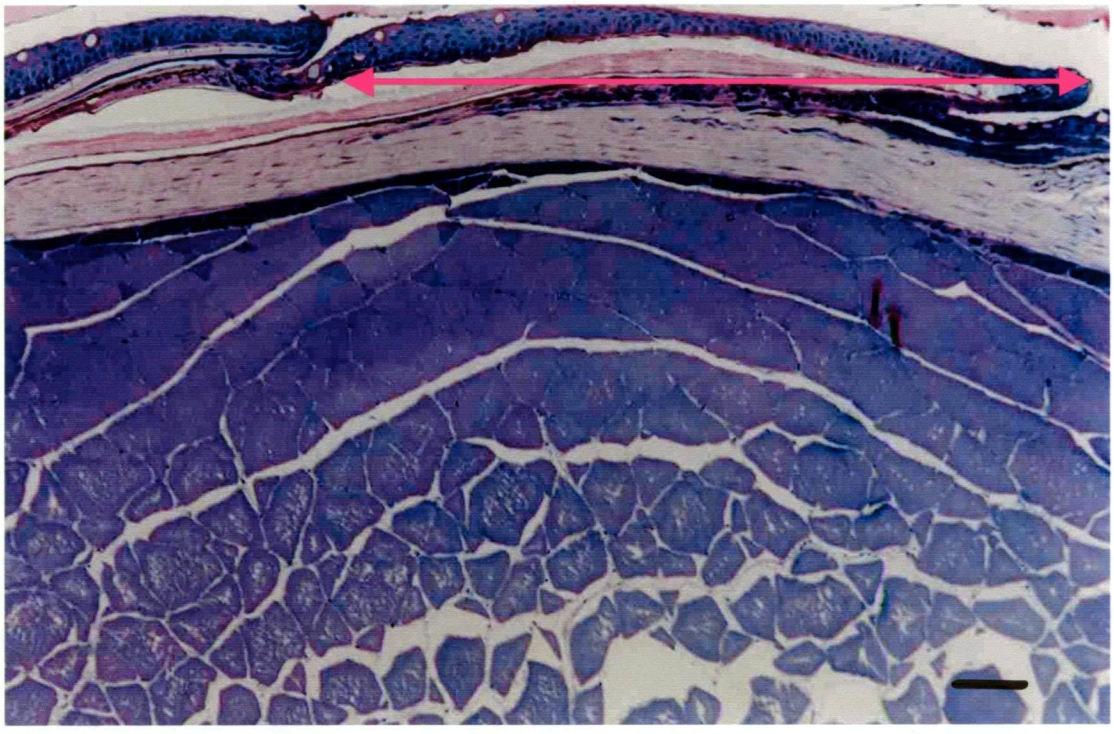


Figure 3.10 Photomicrograph of sand whiting showing a scale unit (arrow) where measurements of epidermal thickness and mucous cell counts were taken. (Polychrome stain, resin section, bar=100 μm)

Table 3.8 Epidermal thickness measurements and mucous cell counts in sand whiting taken from one scale unit. Values are means ± s.e. of 2 replicates. (* Values based on one replicate only due to fish mortality).

	Sampling day	Treatments	
		(+) Control	Cohabitation
Epidermal thickness (μm)			
26 °C	1	61.2 ± 10.6	63.8*
	3	58.6 ± 5.2	33.8*
	6	66.4 ± 6.4	72.8*
	9	49.2 ± 1.1	50.3*
19 °C	1	46.0 ± 4.1	55.9 ± 6.2
	3	39.2 ± 23.6	86.7 ± 18.9
	6	100.6 ± 20.3	86.9*
	9	44.0 ± 8.1	144.1*
Mucous cell counts			
26 °C	1	2.5 ± 0.5	1*
	3	0	0*
	6	0	0*
	9	0.5 ± 0.5	2*
19 °C	1	2.5 ± 0.5	2.0 ± 1.0
	3	0	0.5 ± 0.5
	6	3.0 ± 3.0	0*
	9	3.0 ± 3.0	2.0*

The cells involved in granulomas (mature macrophages, epithelioid cells and multinucleated giant cells) are highly capable of ingesting, degrading and eventual eliminating a persistent infectious agent (Adams 1983). Although some studies (Angelidis *et al.* 1988; Ainsworth *et a*l. 1991; Dexiang and Ainsworth 1991) have reported that phagocytic function is not significantly altered by temperature change and phagocytes can adapt to low temperatures, it seems that the process of killing the infectious agent is highly dependent on environmental temperature. Studies have indicated that intracellular killing is reduced and delayed at low temperature (Sohnle and Chusid 1983; Scott *et al.* 1985; Blazer 1991; Tort *et al.* 1998). It is likely that the phagocyte lytic enzymes involved in intracellular and extracellular killing are temperature-dependent and therefore they require optimum temperature to eliminate the pathogen.

Activation of macrophages is also dependent on cytokines (macrophage activating factor, MAF) produced by T lymphocytes (Hardie *et al.* 1994). Since T lymphocytes are suppressed at nonpermissive temperature, the level of which depends on the fish species, and as they require longer period to undergo homeoviscous adaptation (Bly and Clem 1992), macrophage function is also indirectly affected. In addition, it is likely that some neuro-endocrine factors are involved in the delay and moderate infection response of sand whiting kept at low temperatures. The intensity of cellular infiltrate was significantly lower during the early stage of the infection in temperature-challenged fish but they were able to eventually reach the maximum response exhibited by the fish kept at higher temperature. It is also possible that the stress induced by the drop in temperature affected the infiltration of inflammatory cells. Hormones like glucocorticoids released during stressful events can inhibit MAF uptake by fish macrophages and consequently their killing capacity (Ellis 1981). In mice, Kizaki *et al* (1995) suggested that "suppressive macrophages" proliferate during acute cold stress and this proliferation was related to high glucocorticoid levels. Thereafter, they decreased in number during cold acclimatization when glucocorticoid concentration returned to normal levels.

Temperature could likewise affect the growth and the physiology of infectious agents. Outbreaks of "winter saprolegniasis" in channel catfish are usually

brought about by low temperature-induced immunosuppression of the host and the proliferation of *Saprolegnia* spores at ~10 °C (Bly *et al.* 1993b). In relation to EUS, no outbreaks had ever occurred in the Philippines when the temperature was more than 30 °C. Fraser *et al.* (1992) reported that *A. invadans* isolated from estuarine fish in eastern Australia grew optimally in culture media at 31 °C (bream and mullet) and 22 °C and 25 °C (sand whiting). Fungal isolates from Thailand also exhibited maximum growth at 30 °C but was slow-growing compared with the saprophytic *Aphanomyces* species. Moreover, the Thai fungal isolates could still release spores at 10 °C. In this experiment, *A. invadans* was able to grow and invade the host tissue at 17 °C. Although the rate of hyphal penetration may have been affected to a certain extent by low temperature since there was only a 2-day difference before the fungal hyphae were detected in the sections of fish held at low temperature, the highly invasive hyphae were able to induce severe tissue damage when the immune response of the sand whiting was compromised.

In the second experiment and as in the preliminary trial (Appendix 3), there was no significant change in the epidermal thickness and in the density of mucous cells in sand whiting subjected to temperature decrease and then held at 19 °C with addition of *A. invadans* fungal mats. In channel catfish subjected to rapid temperature drop, the number of epidermal mucous cells decreased in the presence of *Saprolegnia* spores but the mucous cell density returned to normal after 4 days if fungal spores were not present in the experimental system (Quiniou *et al.* 1998). However, in my work, because of the high mortality which resulted to lack of replication in some treatments and minimum number of fish sampled, the quantitative data obtained may not have been reliable enough to draw firm conclusions.

Based on some moribund and dead sand whiting sections examined, the pathogenic *A. invadans* was present and was able to sporulate and grow under the conditions in the experimental tanks. It was likely that the sand whiting were not able to adapt well to freshwater conditions as shown by the heavy mortality during the experiment (pers. comm. Michael Burke, Bribie Island Aquaculture Research Centre) and this was further exacerbated by the gradual decrease in temperature. Thus, even though there were some qualitative pathological changes

in the epidermis, which were unassociated with temperature as they were observed in fish kept at both 26 °C and 19 °C, there was no fungal infection induced in sand whiting and most fish died or were moribund early in the course of the experiment.

The marked epidermal spongiosis observed in abraded fish and non-abraded fish was possibly due to osmotic stress which sometimes lead to bacterial and fungal invasion as was observed histologically in moribund and dead fish . According to Ferguson (1989), severe spongiosis may result to a breakdown in intercellular contact and eventual lifting or loss of epidermis and vesicle formation. These pathological signs were described in ulcerative dermal necrosis (UDN) of salmonids, a condition which occur when salmonids migrate to freshwater environment to spawn, with the skin becoming susceptible to *Saprolegnia* species. The effect of salinity on EUS is beyond the scope of this study but it was also reported (Rodgers and Burke 1981; Virgona 1992; Mohan and Shankar 1994; Callinan *et al.* 1995b) as one of the environmental factors associated with EUS outbreaks in estuarine species, it might be worthy to mention that the histopathological findings in this trial suggest that osmotic stress could contribute to the susceptibility of fish species to *A. invadans* infection.

Based on the results of this experiment, the effects of temperature on the morphology of the epidermis and the distribution of mucous cells in sand whiting subjected to *A. invadans* spores was inconclusive due to other factors affecting the response of the fish to the treatments. Thus, it would be important to investigate this aspect using another fish species which is more robust and where other stressful factors can be excluded in the experimental system.

Chapter Four

Reproduction of EUS in three-spot gourami, *Trichogaster trichopterus* Pallas, subjected to low and fluctuating temperature

4.1 INTRODUCTION

As presented and discussed in Chapter 3, it was not possible to subject sand whiting to the full range of manipulations required to investigate the effects of various water temperature regimes on the expression of EUS. Consequently, a more robust species, the three-spot gourami, *Trichogaster trichopterus*, an obligate air-breathing, freshwater species, was used in the following experiments in order to reduce the compounding effects of factors such as suboptimal DO, salinity stress and physical manipulations.

Howe and Stehly (1998) and Howe *et al.* (1998) reported that experimental infection of rainbow trout and channel catfish with *Saprolegnia* spores were successful, usually resulting in high infection levels, when the fish were subjected to combinations of temperature stress and skin abrasion. It is relevant to those observations that channel catfish have been reported to be more susceptible to *Saprolegnia* infection due to decreased epidermal mucous cell density after a rapid temperature drop (Quiniou *et al.* 1998). Also, fish epidermal responses to different stressful perturbations like organic pollution, heavy metal exposure, acidified (low pH) water and even biological agents or pathogens have been reported. These responses include thinning of the epidermal layer, overproduction of mucus usually leading to depletion of mucous cells, infiltration of leucocytes to the epidermis and change in the mucus composition (Marshall 1979; Zuchelkowski *et al.* 1981; Pottinger *et al.* 1984; Ingersoll *et al.* 1990; Urawa 1992; Iger *et al.* 1992; Iger *et al.* 1994a; Iger *et al.* 1995; Berntssen et al. 1997). Consequently, there are a range of potential insults which could predispose fish to invasion by *A. invadans*. As indicated previously this study is concerned mainly with fluctuations of water temperature.

Two experiments were undertaken using three-spot gouramis in order to determine whether temperature manipulations could induce epidermal changes which could possibly permit the invasion of *A.invadans* into cutaneous and superficial tissues of susceptible fish. The first experiment was initially undertaken to determine whether 3-spot gouramis would be a suitable species for

the experimental conditions. The fish were subjected to rapid temperature drop and skin abrasion and subsequently exposed once to *A. invadans* spores. In the second experiment, the gouramis were subjected to daily fluctuations of temperature to reflect as much as possible, the conditions in natural EUS outbreaks, combined with serial exposures to the pathogen *A. invadans*. The epidermal response was assessed by measuring the epidermal thickness, mucous cell counts based on histochemistry of the mucus content and sacciform cell counts.

4.2 MATERIALS AND METHODS

4.2.1 Fish and husbandry conditions

Experiment I. For the preliminary trial, three-spot gouramis (5-6 cm in total length) obtained from a commercial pet shop were randomly distributed and acclimated in 16 x 40 L glass tanks each with airstones for aeration. They were fed daily to satiation with commercial fish flakes. Water temperature was maintained using immersion heaters. Dissolved oxygen was measured daily, while pH, NH_3-N and NO_2^- levels were measured once during the 14-day acclimation period. Dechlorinated municipal water was used to change the water in all tanks every three days. Mean daily temperature in the tanks was from 25.6 - 26.4 °C, DO level was from 5.6-6.4 mgL^{-1}, pH ranged from 6.6-7.0, while NH_3-N and NO_2^- levels were too low to be detected with the respective measuring kits used.

Experiment II. For this trial, 180 three-spot gouramis (5.7 ± 0.48 cm mean total length) were obtained through the Tasmanian Ornamental Fish Farm, Norwood, Tasmania. The fish were randomly distributed in 40-L glass tanks (10 fish x 18 tanks) but were only filled initially with 10 L of dechlorinated municipal water without aeration. During the 7-day acclimation period, half of the water was replaced every two days with pond water until the fish had adjusted to full-strength pond water. Pond water was delivered to the Aquatic Centre of the

School of Aquaculture in a transportable 500-L fish tank with a fitted tap and lid. Pond water was allowed to stand for the silt to settle for three days in 250-L plastic tanks and then transferred to another 250-L tank with aeration. The fish were fed to satiation daily with commercially prepared fish flakes. The pH(6.9), NH_3-N (undetectable) and NO_2^- (undetectable) levels were measured once while temperature and DO were monitored daily. Mean daily temperature was from 25.8-26.8 °C and mean daily DO ranged from 3.8 to 4.4 mgL^{-1}.

4.2.2 Experimental design

Experiment I. After acclimation, the fish in glass tanks (6 fish x 16 tanks) were allocated into two temperature treatments: 8 tanks of fish were held at 26 °C while the fish in the other 8 tanks were subjected to a rapid temperature drop, from 26 °C to 19 °C within 24 hours and maintained at 19 °C for the duration of the experiment.

Within each temperature regime, the 8 tanks were randomly designated into four different treatments: 1) negative (-) control, no exposure to *A. invadans* spores; 2) positive (+) control, exposure to *A. invadans* spores only; 3) skin abrasion and then exposure to *A. invadans* spores; 4) swaying in air for 2 min and then exposure to *A. invadans* spores. Each treatment was performed in two replicates. In fish subjected to temperature change, the skin abrasion and "swaying in air" experimental treatments/procedures were undertaken after the drop in water temperature.

Experiment II. Of the 18 tanks of fish, the 3-spot gouramis in 9 tanks were held at 26 °C using immersion heaters to maintain the temperature, while the gouramis in the other 9 tanks were kept in a refrigerated room with the temperature maintained at 18-19 °C. However, each of these latter tanks was also provided with an immersion heater set at 26 °C. Daily fluctuations of temperature was accomplished by switching the heaters on and off at 1000 and 2200 hours, respectively.

Three different treatments were employed for each temperature regime: 1) negative (-) control, without any spore exposure; 2) positive (+) control, with spore exposure, and 3) skin abrasion and spore exposure. Each treatment was performed in three replicates. The fish were exposed to *A. invadans* in two ways: by adding either spores to the water or fungal mats which could sporulate within 18-24 hours. To ensure optimum sporulation and survival of spores during the experiment, pond water was used and tank aeration was eliminated to prevent possible early encystment of spores.

4.2.3 Fish exposure to *A. invadans* spores and addition of fungal mats in experimental tanks

Experiment I. Fungal mats and spores of *A. invadans* were produced by methods described in Chapter Two. The fish were not fed 24 hours before spore exposure. On the day of the spore exposure, the fish to be abraded were anesthetised with 80 mgL^{-1} benzocaine and a 1-cm^2 area of the skin on the dorsal region immediately behind the operculum was scarified with the blunt edge of a sterile scalpel blade. The fish were allowed to recover in aerated water and then placed in a 5-L glass beaker with 2 L of pond water with *A. invadans* spores .

For the air-swaying procedure, the fish in each tank were collected and swayed in air for 2 min in a fine-meshed scoop net and returned to their respective tanks and were likewise exposed to fungal spores. The (+) control fish were just placed in 5-L glass beakers and exposed to fungal spores for 24 hours while the (-) control fish were not subjected to any stress or fungal spores. Table 4.1 shows the number of spores added in each experimental beaker. After 24 hours, the fish were returned in their respective tanks with fresh dechlorinated municipal water. Only one fungal spore exposure was performed to ensure that all lesions were initiated on the same day.

Experiment II. Prior to the start of this trial, the *A. invadans* isolate (24P) was passaged once in sand whiting by injecting spores to produce lesions and re-isolating the fungus until an axenic culture was obtained. This axenic fungal

Table 4.1 Experiment I. Approximate spore concentration in the treatment tanks for the 24-hr exposure of three-spot gouramis to *A. invadans* spores in 2000 mL water.

Temperature	Treatment Tanks	Spore Concentration (spores/ml)
26 °C	(+) control, replicate 1	70
	(+) control, replicate 2	73
	air-swaying,replicate 1	65
	air-swaying, replicate 2	60
	skin abrasion, replicate 1	65
	skin abrasion, replicate 2	65
26 °C – 19 °C	(+) control, replicate 1	66
	(+) control, replicate 2	57
	air-swaying,replicate 1	54
	air-swaying, replicate 2	61
	skin abrasion, replicate 1	59
	skin abrasion, replicate 2	64

culture was then used for this trial. Production of 4-day old fungal mats to be placed in the experimental tanks and for *in vitro* sporulation was undertaken every two days for continuous supply of *A. invadans* materials. Spore exposure was done 3-4 hours after the immersion heaters were turned on to make sure that the water temperature was almost equal to that used in the 1-L beakers with 200 ml pond water where *A. invadans* spores had been added. Concentration of spores ranged from 1,000 to 1,800 spores at each exposure day. After a 6-hour exposure, the fish were returned to their respective tanks. Figure 4.1 illustrates the experimental design and treatments for Experiment II while Table 4.2 shows the details of activities undertaken involving the (+) control fish for both temperature regimes during the trial.

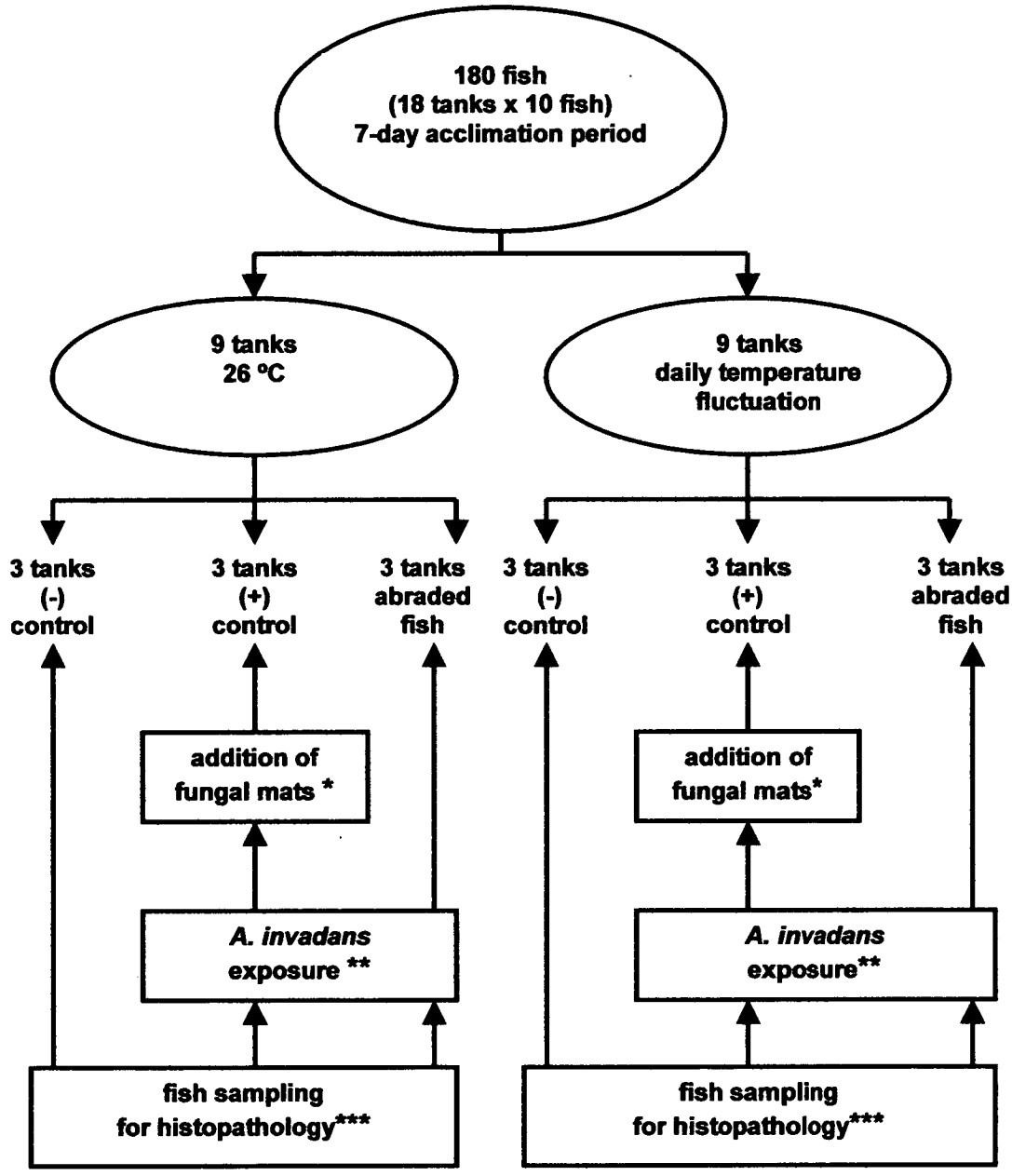
For the scarified fish, the process of skin abrasion was as described for Experiment I. However, the abraded fish were only exposed to fungal spores on day 0 (1,100-1,400 spores/ml) and day 2 (1,300-1,500 spores/ml) of the experiment.

4.2.4 Experimental conditions and tissue sampling

Experiment I. After the spore exposure, aeration and fish feeding were resumed. Any gross signs of infection and mortality were monitored daily. One-half of the water was replaced with dechlorinated municipal water every three days. The fish in tanks subjected to rapid temperature drop were held in a refrigerated room to maintain the temperature at 19 °C while temperature in the other tanks was maintained through the use of immersion heaters.

Sampling was undertaken on day- 2, 4, 6, 8, 10 and 12 post exposure (p.e). Fish, apart from the abraded animals, were sacrificed by hitting their heads with the wooden handle of a laboratory spatula and they were then transversely cut in half. The anterior halves were immediately fixed in 2.5% cacodylate-buffered glutaraldehyde with 2% Alcian blue. The abraded fish were instead euthanased in 200 ppm benzocaine at day-8 p.e. and were fixed in 10% neutral buffered formalin for histopathology.

Figure 4.1 Experimental design for Experiment II.



* Fungal mats were added to the (+) control fish every three days with at least 18 hours allowance for sporulation before fish were exposed to spores.

** Spore exposure in (+) control fish was for 6 hours every 3 days (~1,000-1,800 spores/ml) while abraded fish were only exposed to spores on day 0 and day 2 of the 24-day experiment.

*** Samples for histopathology were collected at days 1,3,6,9,12,15,21 and 24 from the (-) and (+) control fish; abraded fish were sampled at days 0,1,3,9,12,15.

Table 4.2 Schedule of activities for the 3 spot gouramis exposed to *A. invadans* elements [(+) fish only] subjected to 2 temperature regimes* during Experiment II.

Day	A.M.	P.M.
0	Immersion heaters turned on	1st spore exposure (6 hours)
1	D-1 histology sampling; feed & water	add 4-day old fungal mats in tanks
2	change	
3	Feeding	add 4-day old fungal mats in tanks
4	D-3 sampling; feeding & water change	2nd spore exposure
5		
6	DO & temp. readings; feeding	add 4-day old fungal mats in tanks
7	D-6 sampling; feeding & water change	3rd spore exposure
8		
9	DO & temp readings; feeding	add 4-day old fungal mats in tanks
10	D-9 sampling; feeding & water change	4th spore exposure
11		
12	DO and temp readings; feeding	add 4-day old fungal mats in tanks
13	D-12 sampling; feeding & water change	5th spore exposure
14		
15	DO and temp. readings; feeding	add 4-day old fungal mats in tanks
16	D-15 sampling; feeding & water change	6th spore exposure
17		
18	Feeding	add 4-day old fungal mats
19	No sampling done;feeding & water change	7th spore exposure
20		
21	Feeding	add 4-day old fungal mats
22	D-21sampling; feeding & water change	8th spore exposure
23		
24	Feeding	
	D-24 sampling-end of experiment	

*Immersion heaters were turned on (1000 hours) and off (2200 hours) daily for the fluctuating temperature treatments..

Experiment II. During this trial, no feeding was done prior to spore exposures but on all other days, the fish were fed with the commercial fish flakes. Only water temperature and DO levels were monitored closely during the trial, however, no measurements were made within 24 hours after fungal mats were added to minimise water movement which might affect sporulation of the fungus. Samples for histopathology were collected on days 1, 3, 6, 9, 12, 15, 21 and 24 of the experiment. Fish were sacrificed by hitting their head with the wooden handle of a laboratory spatula and they were then fixed in freshwater Davidson's fixative. Whole fish were fixed with the ventral side cut open for proper penetration of fixative.

4.2.5 Histopathology

Experiment I. Glutaraldehyde-fixed tissue samples were trimmed after a 48-hour fixation. A portion of the dorsal muscle, anterior to the dorsal fin was carefully sliced with a sharp, single-edge blade and processed as described in Chapter 3. Resin sections were stained with periodic acid-Schiff (PAS). Cross-sections of abraded fish fixed in 10% neutral buffered formalin were likewise trimmed, decalcified, processed, embedded in paraffin wax, sectioned at 4-5 μm and stained with PAS. Paraffin sections were examined for fungal invasion using an Olympus BH-2 light microscope. Only good and clear resin sections were examined using a Leitz Diaplan light microscope fitted with an eyepiece micrometer to measure the epidermal thickness (10 random measurements in one scale unit as shown in Fig.3.10) and to determine mucous cell concentration along an epidermal length of 250 μm.

Experiment II. For the abraded fish, cross-sections, which include the centre of the scarified area, were trimmed off and fixed further. For the fish with intact skin, the body was cut into 5 pieces (from anterior to posterior part) and placed in histocassettes. All samples were decalcified, processed and embedded in paraffin wax. Sections (5 μm) were cut and then stained using the Alcian blue (AB pH 2.5)-PAS staining technique to distinguish acidic mucous cells from neutral mucous cells.

No quantitative measurements were undertaken on the abraded fish but the sequential features of the lesions were examined using a BH-2 Olympus light microscope. For quantitative skin responses in other fish, three scale units were randomly chosen per fish sample. Five measurements of the epidermal thickness, from the basement membrane to the outer surface of the epidermis, were taken from each scale unit using a light microscope with an eyepiece micrometer and were expressed as mean values of the 15 measurements from each fish. Measurements were taken from the middle portion of the scale unit. Total mucous cells (TMC-regardless of histochemistry reactions), mucous cell with acidic mucopolysaccharide (AB positive-dark blue color), mucous cells with neutral polysaccharide (PAS positive-magenta color), mucous cells with color reactions intermediate between dark blue and magenta and sacciform cells were also counted based on a 250 μm epidermal length of 3 scale units at x400 magnification and values were reported as total counts (from 750 μm) for each fish.

4.2.6 Statistical analysis

Experiment I. A full factorial ANOVA (3-way) was used to analyse the data from the epidermal thickness measurements and mucous cell counts with sampling days, temperature regime (26 °C and rapid drop), fish treatments (negative control, positive control and stressed fish) and their interactions as factors. Data were initially tested for normality and homogeneity of variance (Bartlett's test). The epidermal thickness data were arcsine-square root-transformed while the mucous cell counts were square root-transformed prior to the ANOVA at $P<0.05$. Multiple comparison of treatment means was done using the Tukey-Kramer HSD test.

Experiment II. Prior to ANOVA ($P <0.05$), data were tested for normality and homogeneity of variance. Total mucous cell (TMC) counts, sacciform cell (SC) counts and epidermal thickness (EPI) measurements were analysed without transformations while counts of neutral mucous cells (PAS positive), acidic mucous cells (AB positive) and mucous cells with intermediate reactions

(PAS/AB) were square root-transformed before the full factorial (3-way) ANOVA was performed for each parameter. Comparison of all treatment means was done using the Tukey-Kramer HSD test.

4.3 RESULTS

Experiment I. Water quality during the experimental period was maintained within acceptable limits. The pH, NH_3-N and NO_2^- levels were measured once and were7.3, <0.1 mgL^{-1} and <0.1-2.0 mgL^{-1}, respectively. Dissolved oxygen was only measured twice during the experiment it ranged from 6.1-6.7 mgL^{-1}. There was no mortality during the acclimation period and during the experimental period, even after the handling and spore exposure stress. Except for decreased appetite exhibited by fish held at 19 °C, no significant clinical or external signs were observed in fish with intact skin. The abraded fish kept at 26 °C showed very mild inflammatory reaction which was evident a 2-3 days after the abrasion procedure but the scarified areas started to heal at day 5 p.e. However, for the fish held at 19 °C, the abraded areas only started to show signs of healing at day 8 p.e.

Histopathological examination of intact 3-spot gourami skin showed the normal morphology of the different layers common to most teleost. The outermost layer, the epidermis, is made up of epithelial cells, mucous cells and sacciform cells. The scales originate from the dermis which is made up of a thin *stratum spongiosum* and *stratum compactum* (Fig. 4.2a). No significant pathological change, which could be related with *A. invadans* infection, was observed in the skin and underlying superficial skeletal muscles of fish kept either at 26 °C or 19 °C. For the abraded fish held at 26 °C, slight inflammation was observed but no fungal invasion was seen. However, for some abraded fish kept at 19 °C, mild inflammatory reaction was observed, with very few fungal hyphae in the dermal region but no significant granuloma formation (Fig. 4.2b). Only 2/6 fish (replicate 1) and 3/6 fish (replicate 2) exhibited these signs.

Statistical analysis showed that there was no significant difference in the epidermal thickness measurements taken from the experimental fish except in air-swayed (stressed) fish kept at 19 °C and sampled at day 4 p.e., which had significantly thinner epidermis. Figs. 4.3 (a) and (b) show the trend of the epidermal measurements of fish kept at high and low temperatures during the experimental period.

For the mucous cell counts, significant differences were detected due to temperature, stress treatment, the interaction of temperature and stress treatment, and the interaction of temperature, time (sampling days) and stress treatments. Figs. 4.4 (a) and (b) show the mucous cell counts during the different sampling days. In general, lower counts were obtained from fish kept at 19 °C. Table 4.3 shows the comparison of treatment means analysed using the Tukey-Kramer HSD test at $P < 0.05$.

Overall comparison of means showed significantly lower mucous cell counts in air-swayed fish kept at low temperature at days 4, 8 and 12 p.e and in unstressed fish kept at 19 °C sampled at day 6 p.e. However, only the stressed fish kept at low temperature sampled on day 4 p.e. was significantly different from its respective (-) control group.

Experiment II.

Water quality. Temperature and dissolved oxygen levels were measured before the immersion heaters were turned on. Average daily temperature ranged from 25.0 °C-25.9 °C and DO was from 3.4 mgL^{-1}-5.1 mgL^{-1} for the fish kept at a stable temperature. For the fish subjected to daily fluctuations of temperature, the average minimum temperature was from 19.4 °C-19.7 °C and DO was from 3.5 mgl^{-1}-5.8 mgL^{-1}.

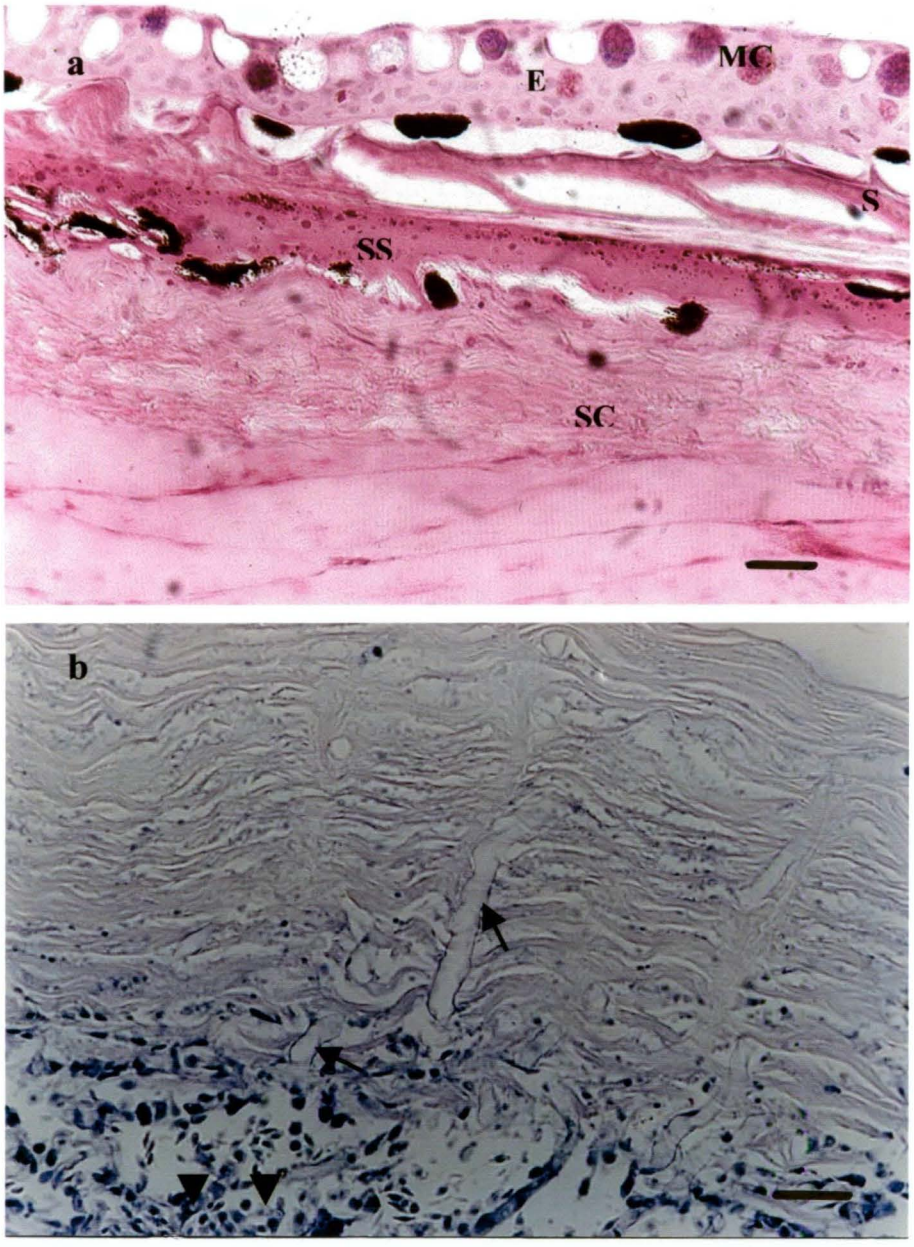
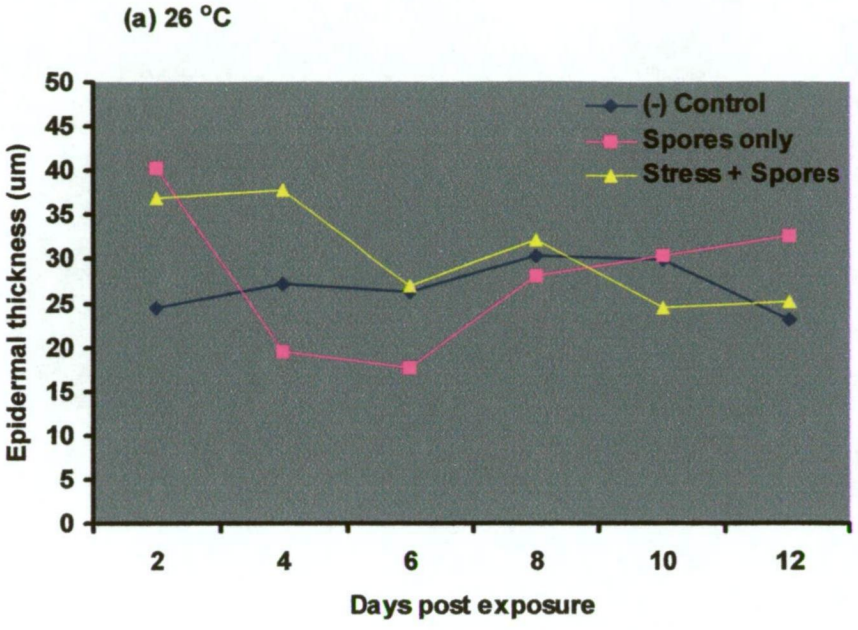


Figure 4.2 (a) Photomicrograph of the skin from an untreated 3-spot gourami: E-epidermis, MC-mucous cells, S-scale, SS-*stratum spongiosum*;SC-*stratum compactum* (PAS, resin embedded,bar= 25 μm). **(b)** Photomicrograph of an abraded gourami exposed to *A invadans* spores after 8 days at 19 °C showing invasive hyphae (arrows) in the dermis and limited inflammatory reaction (arrowheads) in the hypodermal area (PAS, paraffin embedded, bar=25 μm).



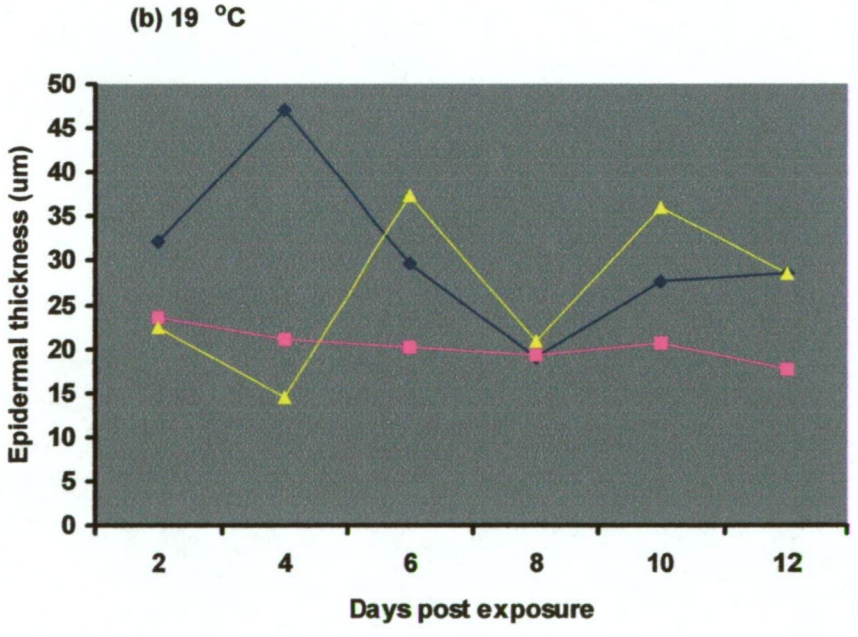


Figure 4.3 Epidermal thickness measurements taken from three-spot gouramis held at (a)26 °C and (b) 19 °C.

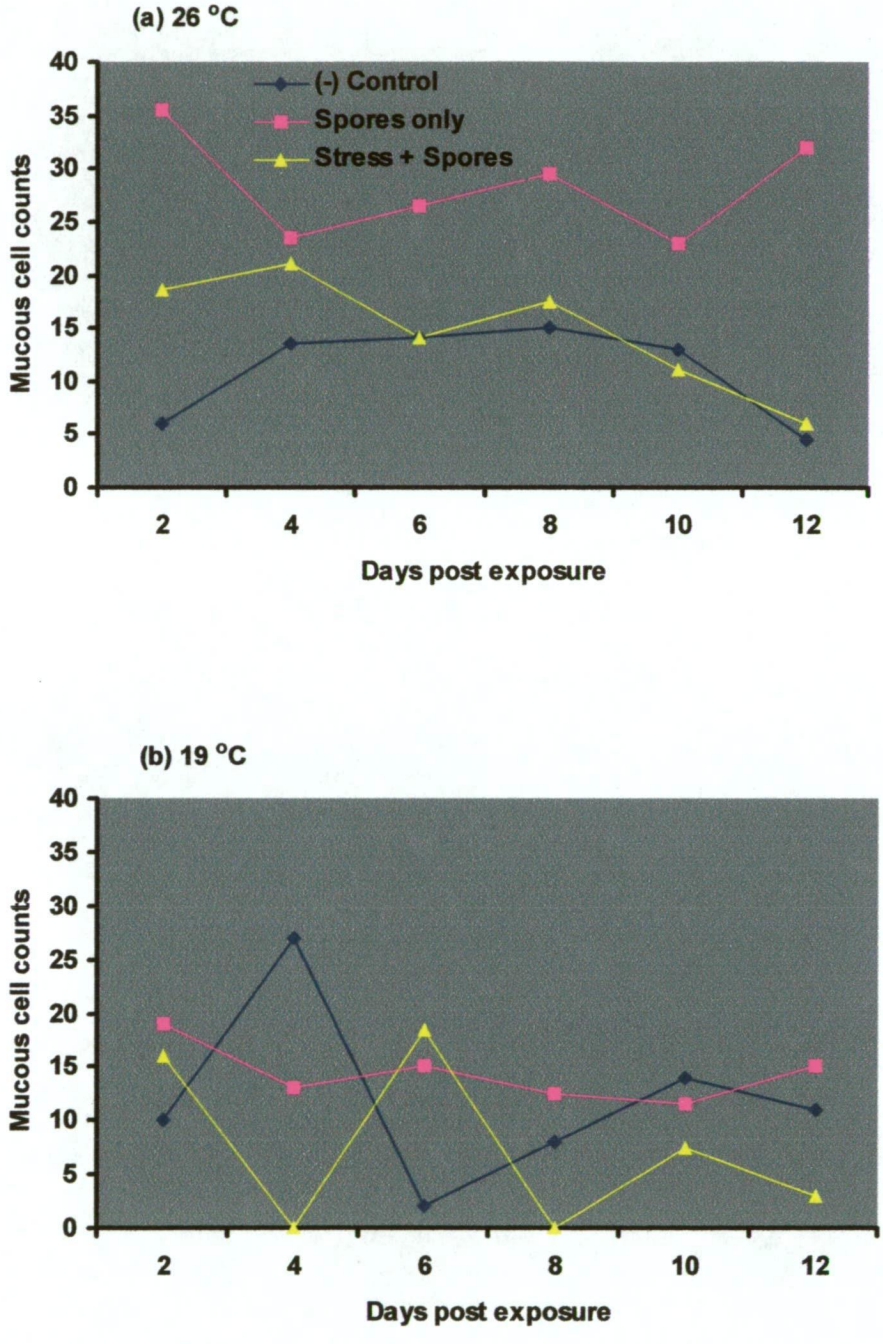


Figure 4.4 Mucous cells counts in 250 μm long epidermis taken from three-spot gouramis held at (a)26 °C and (b) 19 °C.

Table 4.3 Quantitative analysis of the epidermal thickness and mucous cell concentration in three-spot gourami. Values are means ± s.e.; common letters within a column not significantly different by Tukey-Kramer HSD test at $P<0.05$.

Sampling Day	Temperature (°C)	Treatments	Epidermal thickness (μm)	Mucous cell counts
2	26	*(-) Control	24.4^{a}	6.0^{abcd}
		Spores only	40.2 ± 2.4^{a}	35.5 ± 3.5^{e}
		Spores + Stress	36.8 ± 0.2^{a}	18.5 ± 6.5^{bcde}
	19	*(-) Control	32.2^{a}	10.0^{abcde}
		Spores only	23.5 ± 2.3^{a}	19.0 ± 0^{bcde}
		Spores + Stress	22.4 ± 2.6^{a}	16.0 ± 2.0^{bcde}
4	26	(-) Control	27.2 ± 2.2^{a}	13.5 ± 0.5^{abcde}
		Spores only	19.5 ± 4.5^{a}	23.5 ± 9.5^{cde}
		Spores + Stress	37.8 ± 10.3^{a}	21.0 ± 10.0^{bcde}
	19	(-) Control	47.0 ± 1.1^{a}	27.0 ± 11.0^{cde}
		Spores only	21.0 ± 1.8^{a}	13.0 ± 1.0^{abcde}
		Spores + Stress	14.5 ± 0.1^{b}	0^{a}
6	26	(-) Control	26.3 ± 2.5^{a}	14.0 ± 1.0^{abcde}
		Spores only	17.6 ± 3.5^{a}	26.5 ± 0.5^{de}
		Spores + Stress	26.9 ± 10.0^{a}	14.0 ± 4.0^{abcde}
	19	(-) Control	29.6 ± 8.8^{a}	2.0 ± 2.0^{ab}
		Spores only	20.1 ± 3.0^{a}	15.0 ± 3.0^{abcde}
		Spores + Stress	37.4 ± 4.4^{a}	18.5 ± 4.5^{bcde}

*Value from one replicate/section only.

Table 4.3 Continued...

Sampling Day	Temperature	Treatments	Epidermal thickness (μm)	Mucous cell counts
8	26	(-) Control	30.3 ± 4.7^{a}	15 ± 2.0^{abcde}
		Spores only	28.1 ± 2.9^{a}	29.5 ± 3.5^{de}
		Spores + Stress	32.2 ± 4.0^{a}	17.5 ± 1.5^{bcde}
	19	*(-) Control	19.1^{a}	8.0^{abcde}
		Spores only	19.2 ± 2.4^{a}	12.5 ± 0.5^{abcde}
		*Spores + Stress	21.0^{a}	0^{a}
10	26	(-) Control	29.8 ± 0.2^{a}	13.0 ± 1.0^{abcde}
		Spores only	30.4 ± 1.6^{a}	23.0 ± 6.0^{cde}
		Spores + Stress	24.4 ± 5.3^{a}	11.0 ± 5.0^{abcde}
	19	(-) Control	27.6 ± 13.2^{a}	14.0 ± 7.0^{abcde}
		Spores only	20.5 ± 1.7^{a}	11.5 ± 0.5^{abcde}
		Spores + Stress	35.9 ± 9.7^{a}	7.5 ± 0.5^{abcde}
12	26	(-) Control	23.0 ± 1.4^{a}	4.5 ± 4.5^{abcd}
		Spores only	32.6 ± 1.4^{a}	32.0 ± 1.0^{de}
		Spores + Stress	25.2 ± 0.8^{a}	6.0 ± 1.0^{abcd}
	19	(-) Control	28.6 ± 0.2^{a}	11.0 ± 1.0^{abcde}
		Spores only	17.7 ± 1.5^{a}	15.0 ± 4.0^{abcde}
		*Spores + Stress	28.4^{a}	3.0^{abcd}

*Value from one replicate/section only

Fish mortality and clinical signs. No fish died during the acclimation period. Nine anesthetised fish held at stable temperature failed to recover after the abrasion process hence, there were 7 fish x 3 replicates for this treatment. As for the fish subjected to fluctuating temperature, 3 anesthetised fish did not recover after skin abrasion, with 3 replicates x 9 fish for this treatment. At constant temperature, no mortality occurred among (-) control fish and fish exposed to spores during the experimental period. With fluctuating temperature, 3 fish from the (-) control group died (2 fish from replicate 1 and one fish from replicate 3) but there were no dead gouramis from the spore-exposed group.

Table 4.4 shows the mortality in abraded three-spot gouramis subjected to either constant or fluctuating temperature. Mortality first occurred in fish subjected to fluctuating temperature and cumulative mortality was also higher in gouramis exposed to daily temperature change than those fish kept at stable temperature.

Table 4.4 Mortality in skin-abraded three-spot gouramis during the experiment.

Days post exposure	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Constant temp.	0	0	0	0	0	0	0	0	3	2	0	0	0	0	0
Fluctuating temp.	0	0	0	1	0	0	1	2	2	3	1	1	0	0	1

In abraded fish, gross signs of infection were observed on day 3 p.e. with markedly inflamed scarified area, specifically the skin in the periphery of the lesion. By day 9 p.e., fungal hyphae could be seen externally on the lesions but the hyphae appeared collapsed when the fish were taken out of the water (Fig. 4.5). As for the unabraded fish, no significant external manifestations were observed until day 15-24 p.e. when pinpoint discolourations (melanin deposit) and hemorrhages, usually involving one scale along the lateral line or the dorsal area.

On day 24 p.e., two fish (1 fish from replicate 1 and the other from replicate 2) which had been subjected to daily fluctuations of temperature showed mild inflammation in the caudal,ventral area. There were also some pinpoint hemorrhages and the anal fins were eroded.

Histopathology. There was 100% induction of EUS in abraded fish subjected to fluctuating temperatures in 2 replicates and 89% in the other replicate tank. In fish held at high temperature, there was 86% EUS in the 3 replicates. However, samples taken at day15 p.e. from this treatment did not show presence of fungal hyphae in the lesions because of the vigorous response at this temperature.

Histopathological examination of abraded fish showed hemorrhagic areas, cellular infiltration and muscle necrosis at day 1 p.e., for both temperature groups. However, at day 3 p.e., there was greater degree of inflammatory and oedematous reactions in fish held at higher temperature than in fish exposed to temperature variations. No fungal hyphae were seen in the lesions at this stage. However, the lesions from the fish which died on day 4 p.e. showed fungal hyphae in the dermis and limited cellular infiltrate.

Lesions from samples on days 6-15p.e. showed the presence of *A invadans* hyphae which were enveloped within epithelioid cells, as manifested in EUS lesions. At day 6 p.e.,fungal hyphae had invaded the contralateral side with distinct granuloma formation in fish held at high temperature while fish subjected to temperature variations showed less degree of inflammation and minimal granulomas (Fig. 4.6 a and b).

At day 9 p.e., *A. invadans* hyphae had invaded some internal organs such as the kidney and spinal cord. At day 12 p.e., fish kept at high temperature showed the start of resolution while fish exposed to daily temperature variations exhibited greater degree of myonecrosis (Fig. 4.7 a and b).

As for the unabraded fish, the epidermis appeared eroded at day 6 p.e.in some fish subjected to fluctuating temperature which persisted until day 24 p.e.(Fig. 4.8a).

This was exhibited at day 12 by some fish held at high temperature. Examination of the 2 fish sampled on day 24 p.e. (from fluctuating temperature group) showed cellular infiltration in the epidermis, dermis and caudal muscle region (Fig. 4.8b). No signs of fungal hyphae were observed but some rod-shaped bacteria were attached to the inflamed epidermis.

Quantitative histopathology. Statistical analysis did not show any significant difference in the total mucous cell counts (TMC) in all treatments except at day 21 p.e in (-) control fish. Figure 4.10a shows that the TMC for fish subjected to fluctuating temperature (FT) were relatively lower than the fish kept at 26 °C. However, significant differences were detected in the number of PAS(+) mucous cells due to temperature, sampling day and the interaction of temperature and sampling day. Fish held at 26 °C had higher number of PAS (+) mucous cells than fish subjected to FT during the experimental period (Fig. 4.10b).

As for the AB (+) or acidic subpopulation of mucous cells, there was no significant difference detected (Fig. 4.11a) but there was a decreasing trend in the cell number as the experiment progressed. Statistical analysis showed significant difference in the subpopulations of mucous cell with intermediate AB/PAS reactions but was temporally-related rather than temperature effects (Fig. 4.11b).

Sacciform cell counts were significantly lower at day 1 p.e. in fish exposed to spores and held at FT but were not significantly different from the other treatments from day 3-24 p.e. (Fig. 4.12a). Likewise, the epidermal layer did not significantly vary in thickness in all 4 treatments (Fig. 4.12b). Tables 4.5 and 4.6 show the comparison of the treatment means for each parameter in the quantitative skin analysis of three-spot gouramis.

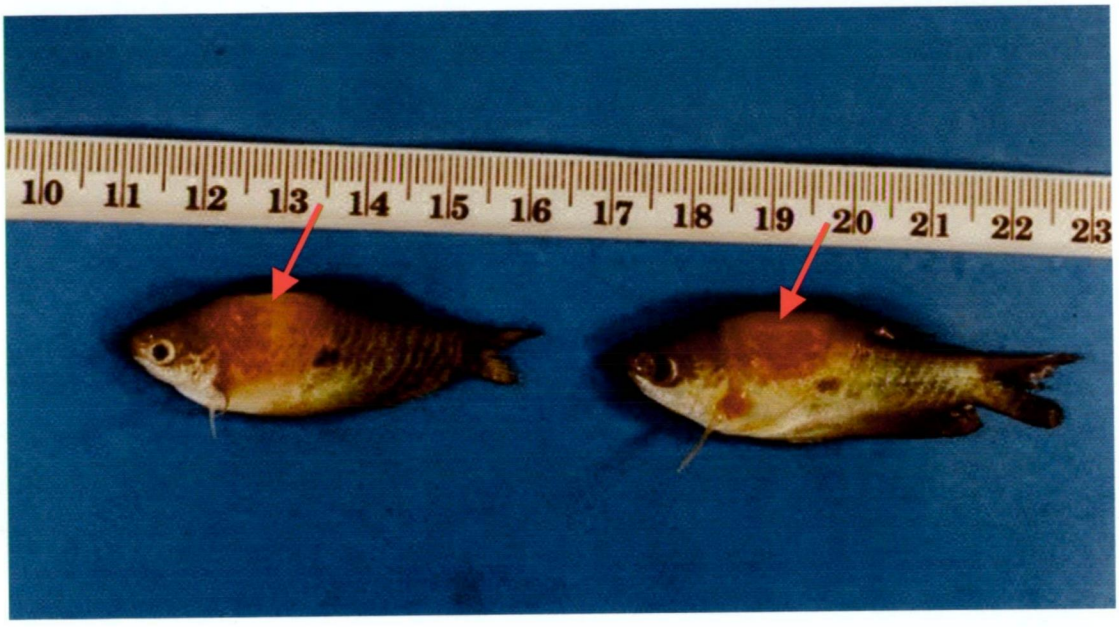


Figure 4.5 Abraded three-spot gourami with *A. invadans* infection (arrows) at day 9 after spore exposure.

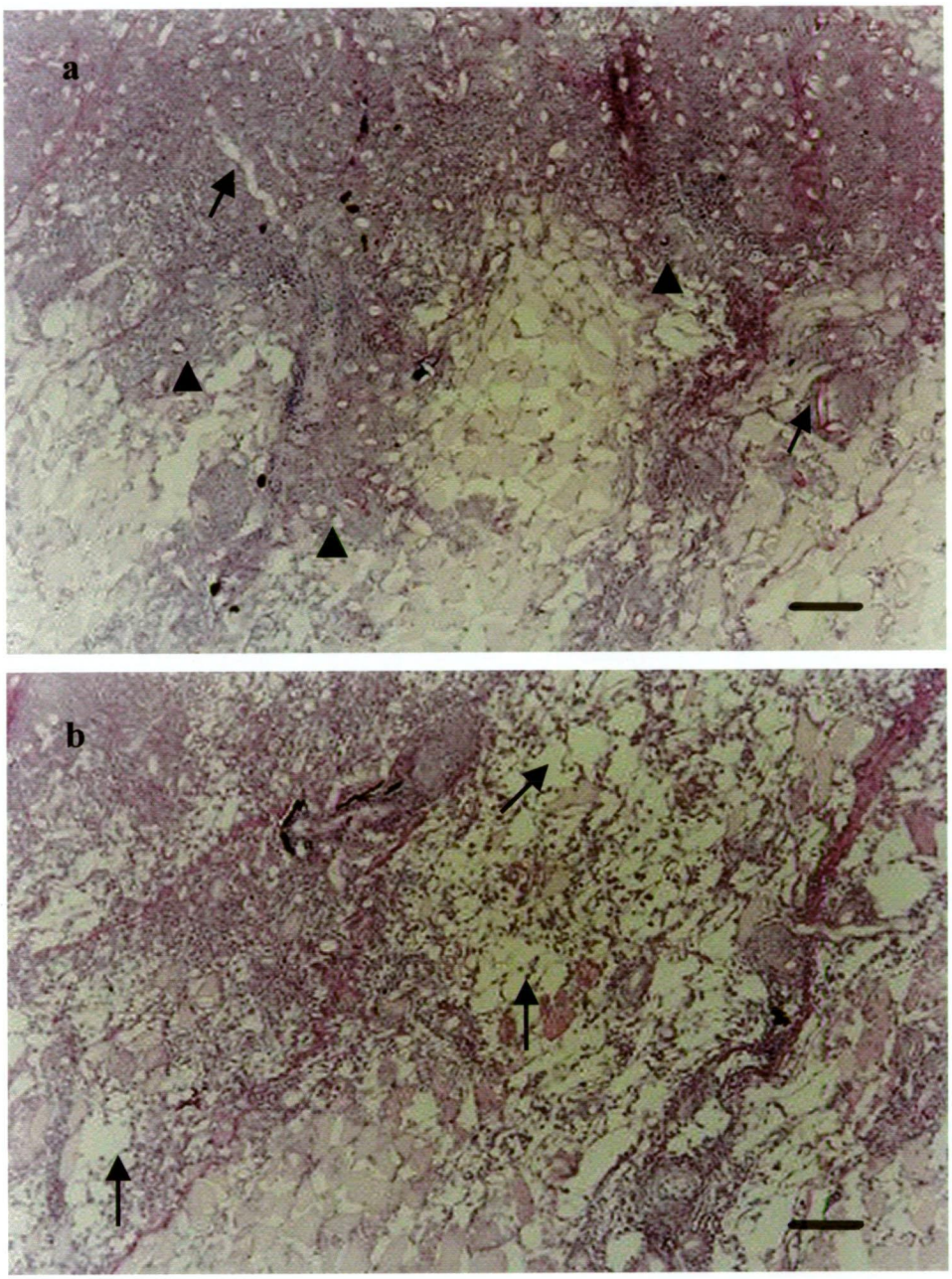


Figure 4.6 Photomicrographs of abraded 3-spot gouramis at day 6 post exposure. **(a)** numerous fungal hyphae (arrows) had invaded the muscle with concomitant cellular infiltration and distinct granulomatous reaction (arrowheads) in fish at 26 °C. **(b)** marked myonecrosis and oedema (arrows) with *A. invadans* infection in fish subjected to fluctuating temperature (FT), granuloma formation not as extensive as in fish kept at 26 °C. (PAS stain, bar =100 μm).

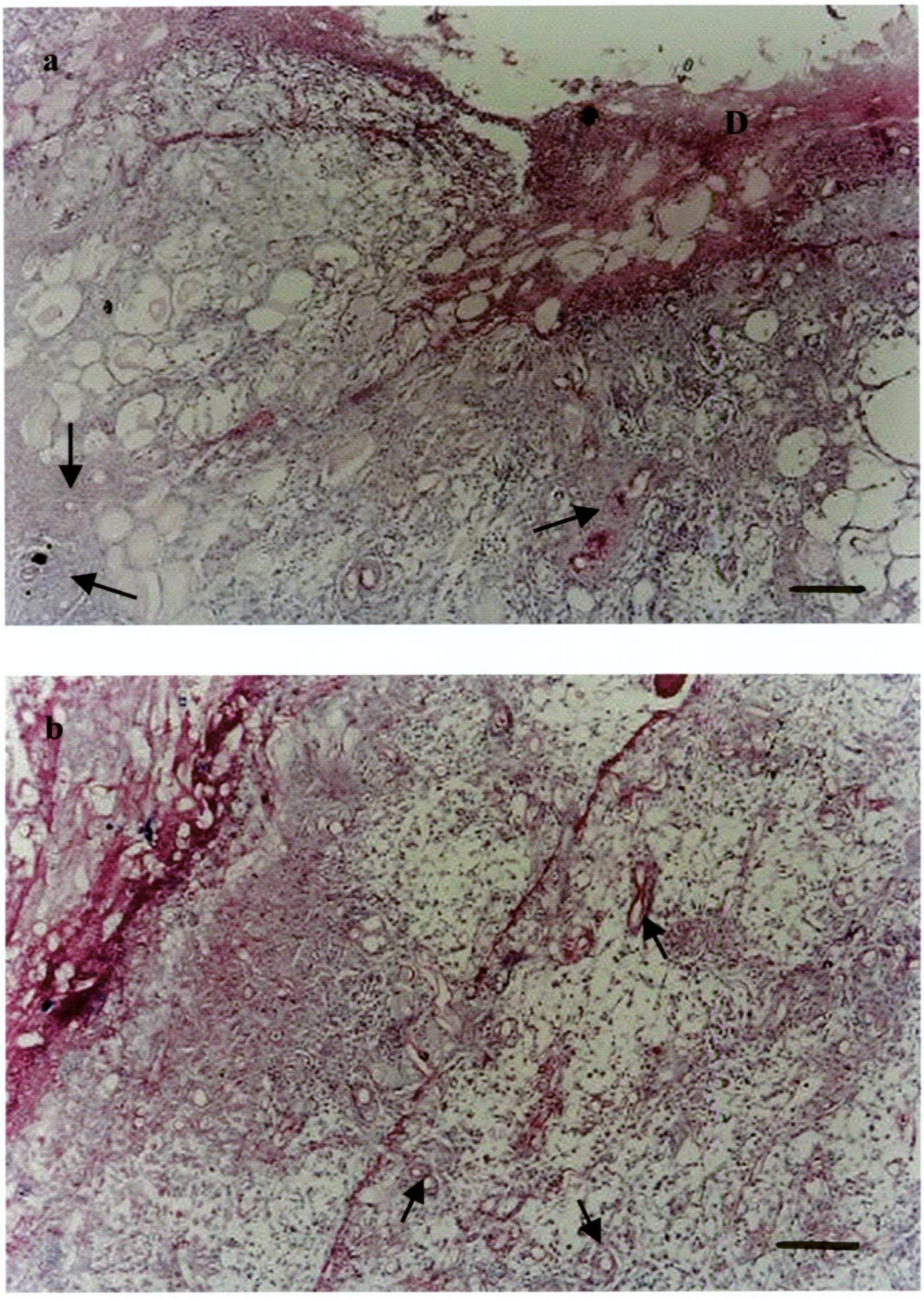


Figure 4.7 Photomicrographs of abraded 3-spot gouramis at day 12 post spore exposure. **(a)** fish kept at 26 °C showed early repair of dermis (D), thick granulomas (arrows) with more fibrotic periphery. **(b)** fish subjected to fluctuating temperature showed extensive muscle degeneration, less intense granulomatous reaction (arrows) than the fish kept at 26 °C. (PAS stain, bar=100 μm).

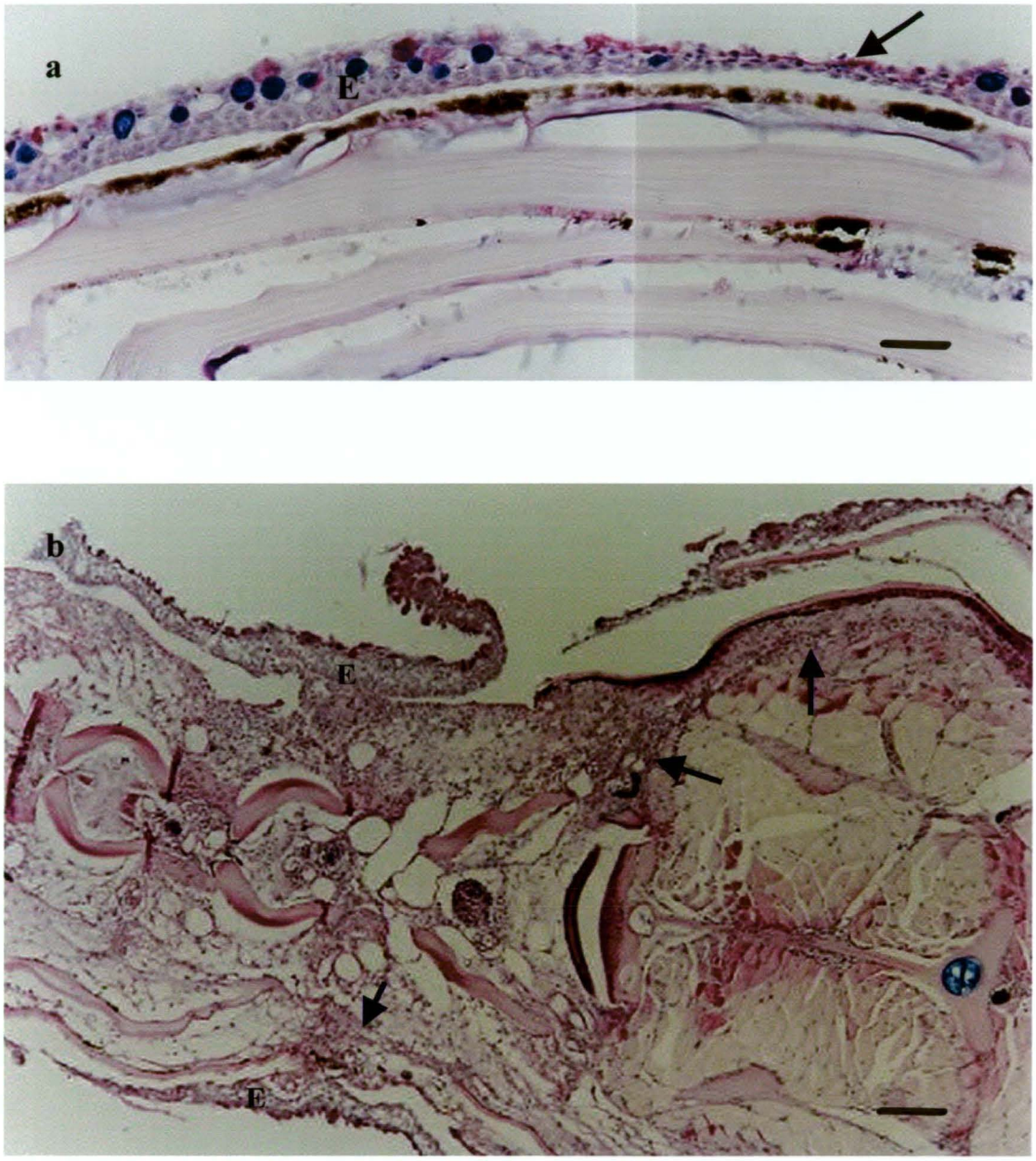


Figure 4.8 Photomicrographs of unabraded 3-spot gouramis subjected to FT showed **(a)** eroded (arrow) portion of the epidermis (E) at day 12 post exposure (AB/PAS stain, bar=25 μm); **(b)** at day 24 of the experiment, two fish had infected caudal area with the epidermal (E) layer and subcutaneous tissues markedly infiltrated (arrows) with inflammatory cells. (PAS stain, bar=100 μm).

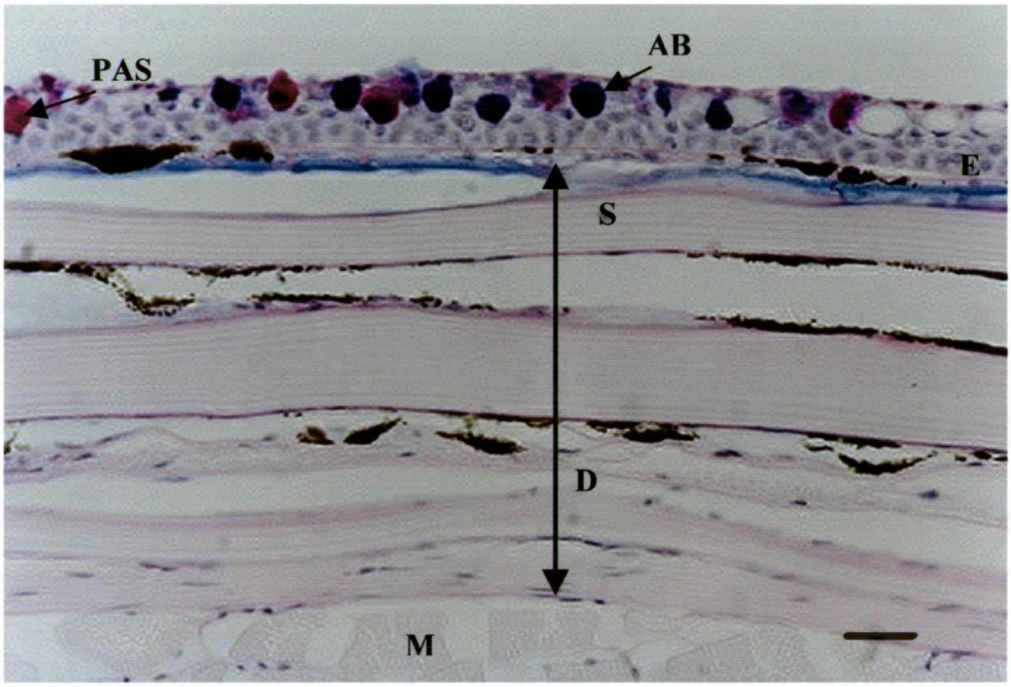
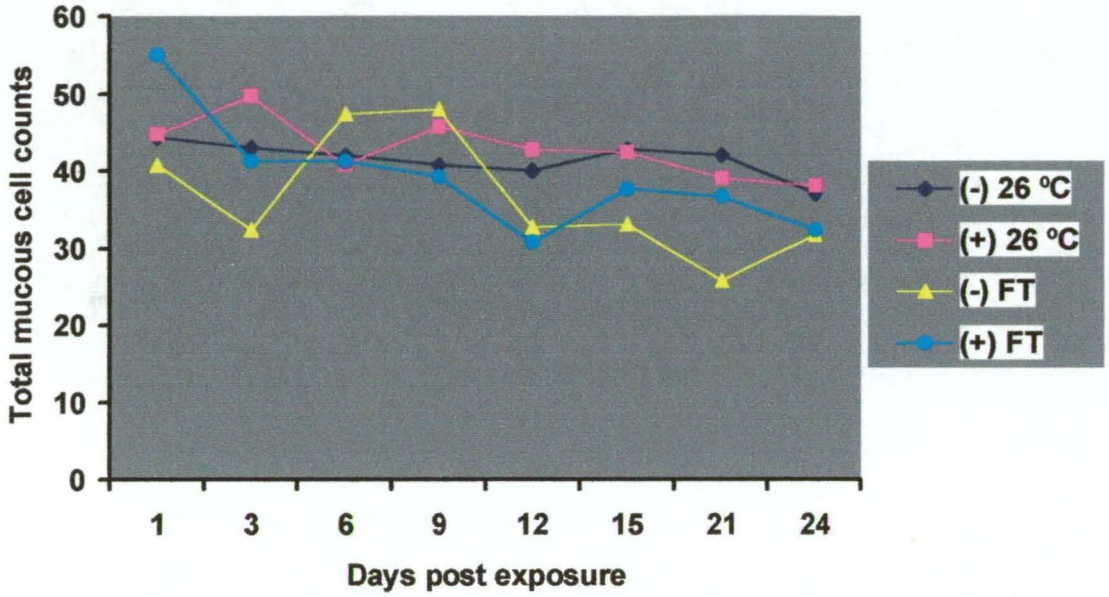


Figure 4.9 Photomicrograph of 3-spot gourami skin showing the AB/PAS stain reactions of epidermal mucous cells. PAS-mucous cell with neutral mucopolysaccharide; AB-mucous cell with acidic mucopolysaccharide; E-epidermis; S- scale, D-dermis, M-muscle. (AB/PAS stain, bar=25 μm).

(a)



(b)

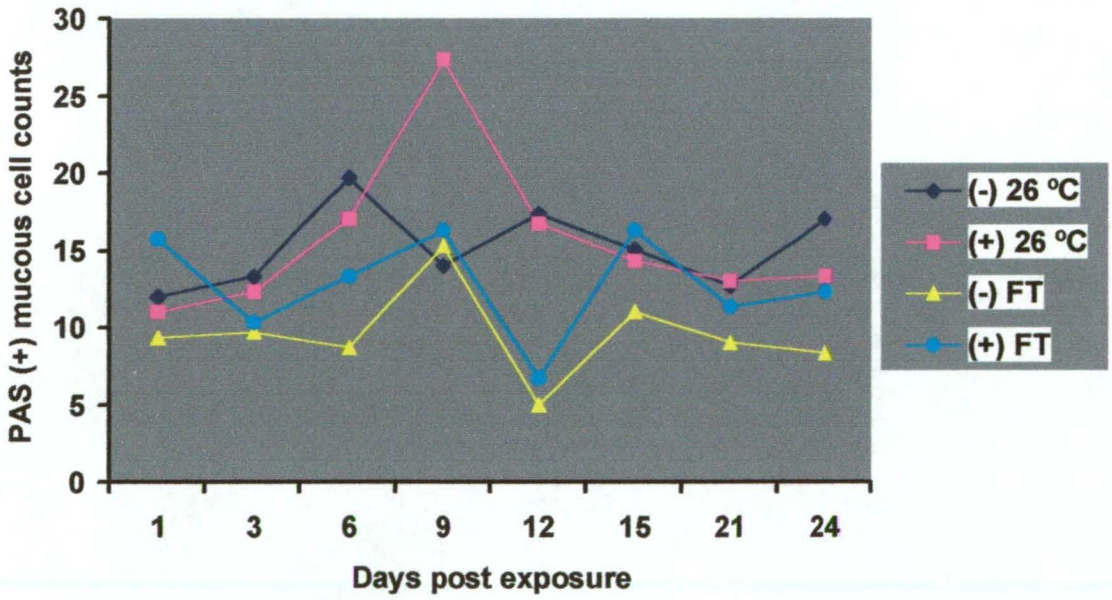
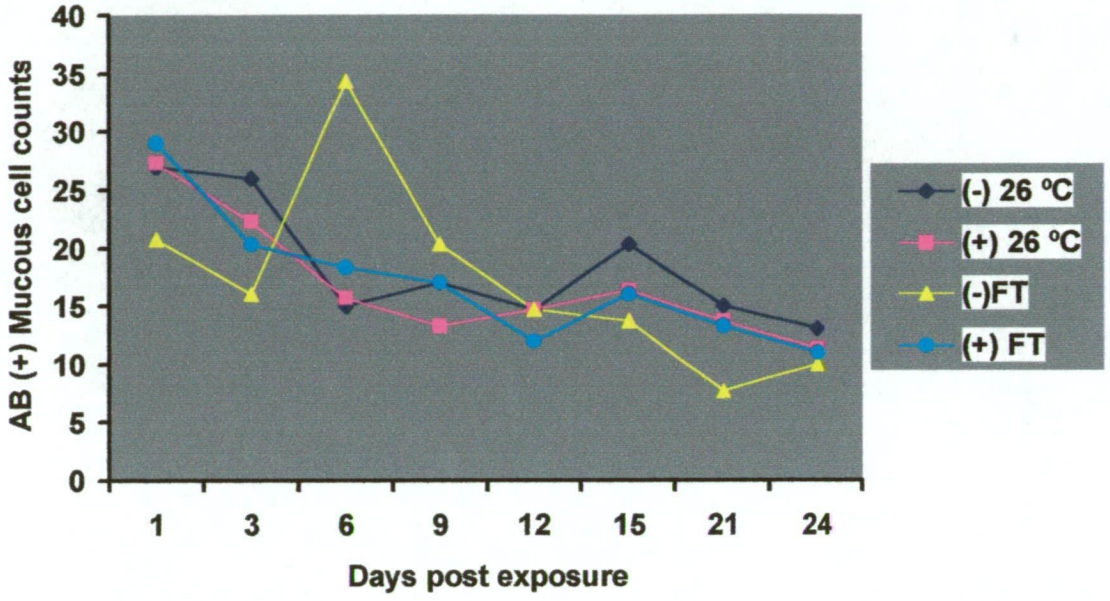


Figure 4.10 **(a)** Total mucous cell counts and **(b)** neutral mucous cell counts in 250 μm long epidermis of each 3 scale units of 3-spot gourami.

(a)



(b)

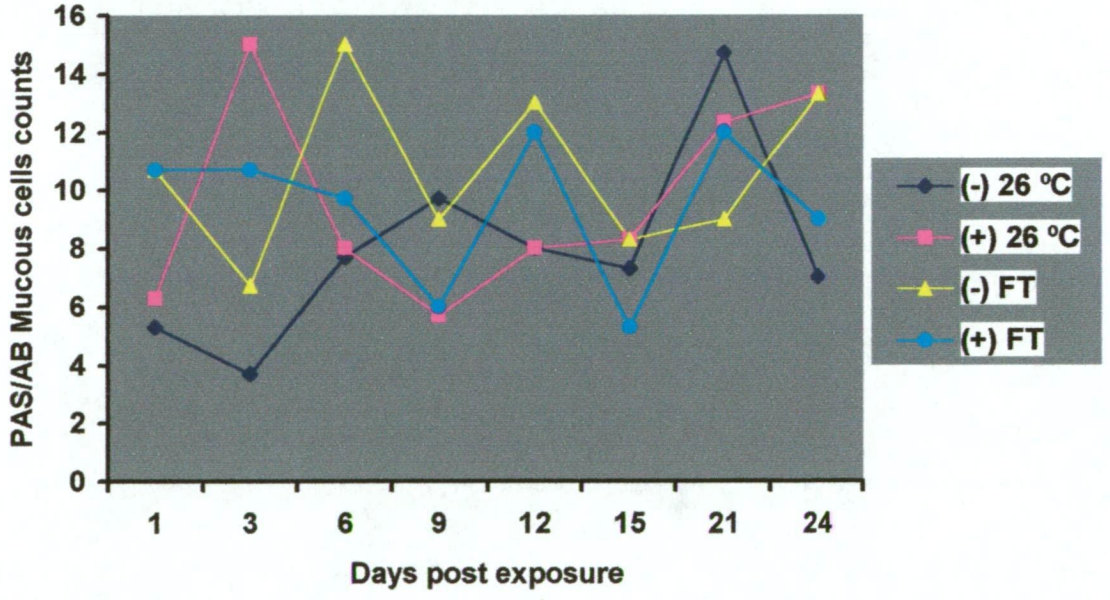
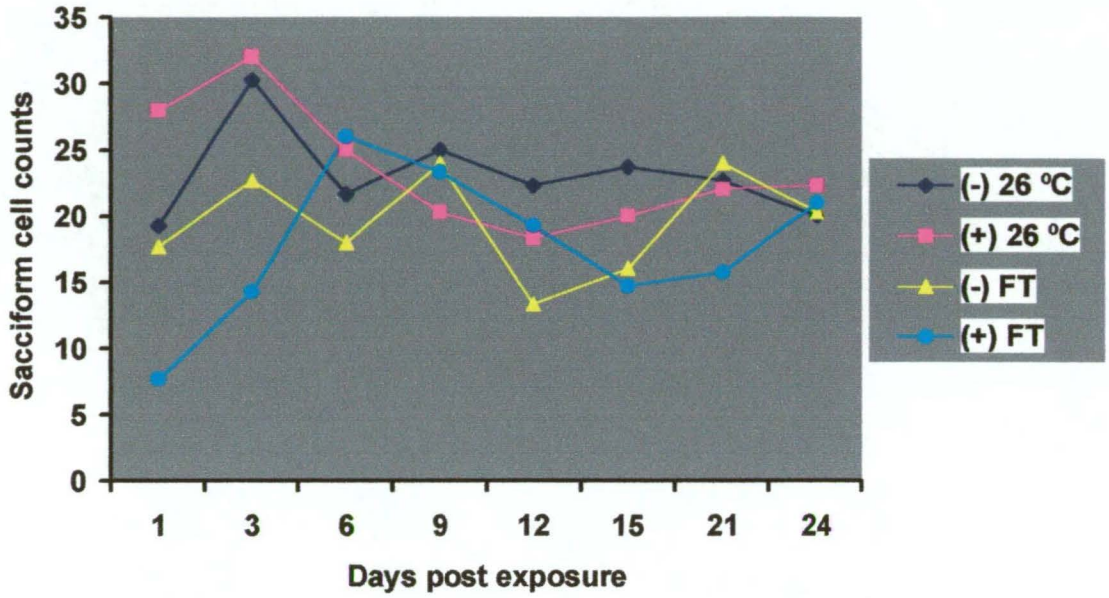


Figure 4.11 **(a)** Acidic mucous cells counts and **(b)** mucous cells counts with intermediate color reactions in 250 long epidermis of each 3 scale units of three-spot gourami.

(a)



(b)

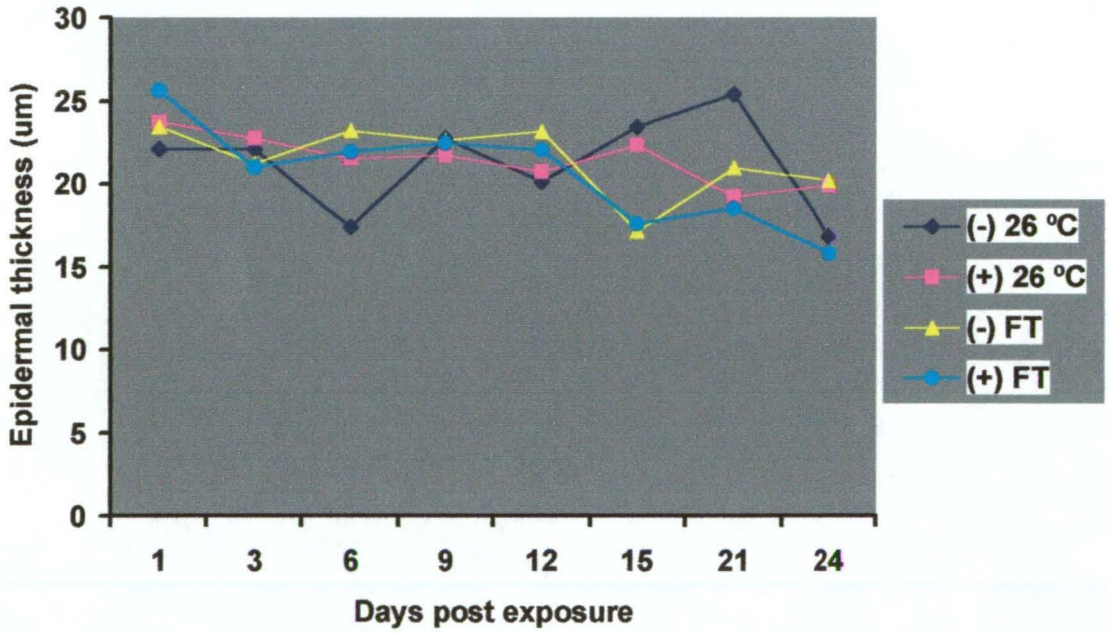


Figure 4.12 **(a)** Sacciform cell counts and **(b)** epidermal thickness measurements from experimental three-spot gouramis.

Table 4.5 Quantitative analysis of the epidermal thickness, TMC and SC in three-spot gourami. Values are means ± s.e.; common letters within a column not significantly different by Tukey-Kramer HSD test at $P<0.05$.

Days post exposure	Treatments	Epidermal thickness (μm)	Total mucous cell counts	Sacciform cell counts
1	(-) 26 °C	22.1 ± 2.3^{a}	44.3 ± 5.4^{a}	19.3 ± 2.7^{ab}
	(+) 26 °C	23.7 ± 1.2^{a}	44.7 ± 6.4^{a}	28.0 ± 5.2^{ab}
	(-) FT	23.4 ± 1.5^{a}	40.7 ± 7.7^{a}	17.7 ± 3.3^{ab}
	(+) FT	25.6 ± 0^{a}	55.0 ± 6.0^{a}	7.5 ± 3.5^{b}
3	(-) 26 °C	22.1 ± 0.8^{a}	43.0 ± 3.6^{a}	30.3 ± 3.2^{a}
	(+) 26 °C	22.7 ± 3.4^{a}	49.7 ± 7.3^{a}	32.0 ± 2.3^{a}
	(-) FT	21.2 ± 1.1^{a}	32.3 ± 3.9^{a}	22.7 ± 4.7^{ab}
	(+) FT	21.0 ± 2.2^{a}	41.3 ± 2.0^{a}	14.3 ± 3.0^{ab}
6	(-) 26 °C	17.4 ± 2.4^{a}	42.0 ± 1.0^{a}	21.5 ± 1.5^{ab}
	(+) 26 °C	21.5 ± 1.5^{a}	40.7 ± 7.6^{a}	25.0 ± 2.6^{ab}
	(-) FT	23.2 ± 1.0^{a}	47.3 ± 1.8^{a}	18.0 ± 1.2^{ab}
	(+) FT	21.9 ± 1.9^{a}	41.3 ± 3.9^{a}	26.0 ± 1.5^{ab}
9	(-) 26 °C	22.7 ± 0.2^{a}	40.5 ± 5.5^{a}	25.0 ± 3.0^{ab}
	(+) 26 °C	21.7 ± 1.7^{a}	45.7 ± 3.0^{a}	20.3 ± 6.7^{ab}
	(-) FT	22.6 ± 0.3^{a}	48.0 ± 5.0^{a}	24.0 ± 4.0^{ab}
	(+) FT	22.4 ± 0.2^{a}	39.3 ± 2.6^{a}	23.3 ± 3.5^{ab}
12	(-) 26 °C	20.1 ± 1.9^{a}	40.0 ± 4.2^{a}	22.3 ± 1.8^{ab}
	(+) 26 °C	20.7 ± 1.4^{a}	42.7 ± 4.5^{a}	18.3 ± 3.4^{ab}
	(-) FT	23.1 ± 2.2^{a}	32.7 ± 7.5^{a}	13.3 ± 0.3^{ab}
	(+) FT	22.0 ± 2.2^{a}	30.7 ± 7.5^{a}	19.3 ± 3.2^{ab}
15	(-) 26 °C	23.4 ± 2.2^{a}	42.7 ± 3.7^{a}	23.7 ± 2.6^{ab}
	(+) 26 °C	22.3 ± 2.5^{a}	42.3 ± 4.9^{a}	20.0 ± 3.5^{ab}
	(-) FT	17.6 ± 1.0^{a}	33.0 ± 0.6^{a}	16.0 ± 1.0^{ab}
	(+) FT	17.1 ± 0.3^{a}	37.7 ± 3.7^{a}	14.7 ± 3.0^{ab}
21	(-) 26 °C	25.4 ± 3.8^{a}	42.0 ± 1.0^{a}	22.5 ± 13.5^{ab}
	(+) 26 °C	19.7 ± 0.7^{a}	39.0 ± 2.6^{a}	22.0 ± 2.1^{ab}
	(-) FT	20.9 ± 1.0^{a}	25.7 ± 2.9^{b}	24.0 ± 1.2^{ab}
	(+) FT	18.5 ± 1.6^{a}	36.7 ± 5.0^{a}	15.7 ± 3.2^{ab}
24	(-) 26 °C	16.8 ± 1.1^{a}	37.0 ± 5.0^{a}	20.0 ± 2.3^{ab}
	(+) 26 °C	19.9 ± 1.1^{a}	38.0 ± 3.0^{a}	22.3 ± 1.7^{ab}
	(-) FT	20.2 ± 1.0^{a}	31.7 ± 1.8^{a}	20.3 ± 4.3^{ab}
	(+) FT	15.8 ± 1.5^{a}	32.3 ± 4.5^{a}	21.0 ± 2.1^{ab}

Table 4.6 Quantitative analysis of PAS (+), AB (+) and AB/PAS mucous cell concentrations in three-spot gourami. Values are means ± s.e.; common letters within a column not significantly different by Tukey-Kramer HSD test at $P<0.05$.

Days post exposure	Treatments	PAS (+) mucous cell counts	AB (+) mucous cell counts	AB/PAS mucous cell counts
1	(-) 26 °C	12.0 ± 0.6^{ab}	5.3 ± 3.5^{a}	27.0 ± 3.5^{ab}
	(+) 26 °C	11.0 ± 0.6^{ab}	6.3 ± 1.4^{a}	27.3 ± 4.7^{ab}
	(-) FT	9.3 ± 0.3^{b}	10.7 ± 3.8^{a}	20.7 ± 4.6^{abcd}
	(+) FT	15.5 ± 2.5^{ab}	10.5 ± 1.5^{a}	29.0 ± 7.0^{ab}
3	(-) 26 °C	13.3 ± 0.9^{ab}	3.7 ± 1.2^{a}	26.0 ± 1.7^{abc}
	(+) 26 °C	12.3 ± 1.2^{ab}	15.0 ± 3.8^{a}	22.3 ± 2.8^{abcd}
	(-) FT	9.7 ± 1.7^{b}	6.7 ± 2.7^{a}	16.0 ± 2.3^{bcde}
	(+) FT	10.3 ± 3.2^{b}	10.7 ± 0.9^{a}	20.3 ± 2.6^{abcd}
6	(-) 26 °C	19.5 ± 1.5^{a}	7.5 ± 2.5^{a}	15.0 ± 3.0^{bcde}
	(+) 26 °C	17.0 ± 3.5^{a}	8.0 ± 3.2^{a}	15.7 ± 2.3^{bcde}
	(-) FT	8.7 ± 3.8^{b}	15.0 ± 5.1^{a}	34.3 ± 0.9^{a}
	(+) FT	13.3 ± 3.0^{ab}	9.7 ± 4.7^{a}	18.3 ± 1.8^{abcde}
9	(-) 26 °C	14.0 ± 1.0^{ab}	9.5 ± 1.5^{a}	17.0 ± 3.0^{abcde}
	(+) 26 °C	27.3 ± 2.2^{a}	5.7 ± 3.2^{a}	13.3 ± 3.3^{bcde}
	(-) FT	15.3 ± 2.0^{ab}	9.0 ± 5.0^{a}	20.3 ± 2.0^{abcd}
	(+) FT	16.3 ± 4.5^{ab}	6.0 ± 1.5^{a}	17.0 ± 3.5^{bcde}
12	(-) 26 °C	17.3 ± 2.0^{a}	8.0 ± 2.9^{a}	14.7 ± 4.3^{bcde}
	(+) 26 °C	16.7 ± 4.6^{ab}	8.0 ± 5.1^{a}	14.7 ± 1.4^{bcde}
	(-) FT	5.0 ± 2.6^{b}	13.0 ± 3.5^{a}	14.7 ± 2.6^{bcde}
	(+) FT	6.7 ± 2.2^{b}	12.0 ± 1.5^{a}	12.0 ± 2.0^{cde}
15	(-) 26 °C	15.0 ± 3.2^{ab}	7.3 ± 1.7^{a}	20.3 ± 0.3^{abcd}
	(+) 26 °C	14.3 ± 2.6^{ab}	8.3 ± 2.3^{a}	16.3 ± 2.3^{bcde}
	(-) FT	11.0 ± 2.0^{ab}	8.3 ± 1.2^{a}	13.7 ± 1.3^{bcde}
	(+) FT	16.3 ± 3.9^{ab}	5.3 ± 0.3^{a}	16.0 ± 0.6^{bcde}
21	(-) 26 °C	12.5 ± 2.5^{ab}	14.5 ± 4.5^{a}	15.0 ± 1.0^{bcde}
	(+) 26 °C	13.0 ± 0.6^{ab}	12.3 ± 2.3^{a}	13.7 ± 2.0^{bcde}
	(-) FT	9.0 ± 1.2^{b}	9.0 ± 2.0^{a}	7.7 ± 0.7^{e}
	(+) FT	11.3 ± 2.9^{ab}	12.0 ± 1.5^{a}	13.3 ± 0.9^{bcde}
24	(-) 26 °C	17.0 ± 2.1^{a}	7.0 ± 2.5^{a}	13.0 ± 0.6^{bcde}
	(+) 26 °C	13.3 ± 1.8^{ab}	13.3 ± 0.7^{a}	11.3 ± 2.0^{de}
	(-) FT	8.3 ± 1.8^{b}	13.3 ± 1.8^{a}	10.0 ± 1.5^{de}
	(+) FT	12.3 ± 1.2^{ab}	9.0 ± 5.0^{a}	11.0 ± 1.0^{de}

4.4 DISCUSSION

The results from both experiments showed that neither a rapid drop in temperature nor daily temperature fluctuation for 24 days could incur sufficient epidermal damage in three-spot gourami that could allow the invasion of the pathogenic *A. invadans*. Lesions typical of EUS were induced only in gouramis which were skin-abraded. Howe *et al.* (1998) used a combination of physical abrasion and low temperature stress to produce saprolegniasis in channel catfish with an infection rate of 87% at day 7 and 100% at day 14, with 90% mortality. However, physical abrasion alone, which was done by draining the tanks daily and allowing the fish to swim against an abrasive surface, failed to induce saprolegniasis while low-temperature shock alone was likewise not adequate since infection was only 3.3% at day 7 and 10% at day 14. Similarly, Howe and Stehly (1998) used the same methods to induce saprolegniasis in rainbow trout but instead of low-temperature shock, the water temperature was increased. Temperature stress alone or the physical abrasion technique was not adequate to induce *Saprolegnia* infection in the experimental fish but 77.8% of the fish were infected when the combination of physical abrasion and temperature shock was employed. They attributed these results mainly to the pathogenicity of *Saprolegnia* species they used and possibly to the experimental fish being highly resistant to the stress factors used in the trials.

In the first experiment in this study, the rapid drop in temperature and subsequent 24-hr exposure to *A. invadans* spores failed to initiate lesions in 3-spot gouramis. The quantitative analysis of the epidermis showed that such treatment did not induce any significant change in the epidermal thickness and mucous cell counts during the 12-day experimental period hence the fungal spores were not able to attach or invade the fish's skin. However, fish that were stressed by air swaying and low temperature showed a significantly thinner epidermis and low mucous cell counts at day 4 p.e. At day 6 p.e., fish for this treatment had recovered, with thicker epidermis and higher mucous cells counts than the (-) control fish. It is apparent that epidermal thickness can be related to the concentration of mucous

cells in the epidermis as shown by the 12-day trends in (-) control and air-swayed fish held at 19 °C. But as in the other treatment, no *A. invadans* infection was induced in gouramis subjected to this experimental treatment. It might be that even if there were some epidermal changes induced by the treatment during the first few days of the experiment, the spore concentration that was used during the single fungal exposure might have been inadequate. On the other hand, continuous exposure to *A. invadans* spores may not likewise be effective since the quantitative analysis of the epidermis and mucous cell counts showed that the fish's skin was able to recover from physical stress and adapt to the temperature after 4 days. This type of response was reported by Iger *et al* (1994a; 1994b;1995) who showed that in fish exposed to acidified water, high–thermal stress or polluted water, the initial mucus production and exhaustion was followed by an acclimation phase manifested by restoration of mucous cell population and other epidermal reparative process at day 7 after exposure to the stressors.

It is also possible that the fungal isolate used might not have been as virulent as when it was initially obtained. The results from the abrasion treatment seem to support this assumption. There was low infection prevalence in Experiment I as compared to the high EUS induction rate in Experiment II when the fungal isolate was passaged and reisolated from an artificially-infected sand whiting. Moreover, no fungal invasion was observed in any abraded fish held at 26 °C in the first experiment whereas in Experiment II, *A. invadans* infection was induced in abraded fish held at 26 °C and in fish subjected to a daily minimum temperature of 19 °C. It is also likely that the type of experimental water used had some effects on the growth and physiology of the spores since in Experiment I, the fish were transferred to their respective tanks with dechlorinated tap water after spore exposure in pond water. It has been determined in a preliminary *in vitro* trial that *A. invadans* isolates sporulate and grow better in pond water than in tap water (Appendix 1) and in a tank experiment similar to Experiment II where dechlorinated municipal water was used, infection rate was also very low in abraded fish (Catap and Munday, unpub. data).

In the second experiment, the potential effects of dechlorinated water on *A. invadans* had been avoided by using pond water and the infectivity of the 24P isolate was likely increased by the single passage done in sand whiting as shown by 89%-100% fungal infection in abraded fish subjected to fluctuating temperature. The concentration of spores used was also higher for this trial which possibly improved the infection rate in both temperature treatments. The higher infection rate in fish subjected to fluctuating temperature could be attributed to the temperature variations. At day 15 p.e., the abraded area in the sampled gouramis held at 26 °C had already healed, the early resolution possibly preventing further infection even if there were spores being spread by infected fish while tissue repair took a bit longer for fish kept at varying temperatures, allowing the infection of other abraded fish which were not initially infected at day 0 and day 2 of the experiment. The additional stress imposed by daily variations in temperature also caused the higher mortality of infected fish compared to the fish held at constant temperature.

As for the unabraded fish, the fluctuating temperature, like rapid temperature drop, failed to induce sufficient skin damage to enable the *A. invadans* spores to infect the fish based on the different skin parameters assessed during the experimental period. There was no significant change in the epidermal thickness and in the total mucous cell counts. However, there were lower TMC in fish subjected to fluctuating temperature from day 12 to day 24 p.e. which could be related to the significantly less number of PAS (+) mucous cells during the same period and to some extent, although not significant, to the decreasing trend in the number of AB(+) cells. Iger *et al.* (1994a) reported that newly-formed mucous cells are basically PAS(+) or with neutral mucopolysaccharides. It is likely that instead of new mucous cells being formed, new epithelial cells were produced to repair the eroded epidermal cells. It is also likely that mucus was being constantly released due to treatment stress as indicated by the bimodal trend in the AB/PAS cell counts but the generation time for the differentiation of the mucous cells when temperature fluctuates could take longer than when the temperature is high and stable. It is possible that prolonged exposure to such temperature

stress could have led to mucus depletion. However, there was still sufficient mucus production during the 24-day experimental period which probably prevented skin damage and attachment of the zoospores. Urawa (1992) reported that the attachment of the ectoparasite *Ichthyobodo necator* on the skin of chum salmon, *Oncorhynchus keta*, led to an initial discharge of mucus which was AB (+) and after 5 weeks, mucous cell concentration increased with concomitant increase in PAS (+) cells and decrease in the parasite load. The control chum salmon had predominantly acidic (AB positive) mucous cells throughout the experiment. Alcian blue (+) mucous cells at pH 2.5 usually contain sulphate-free sialic acid while at pH 1.0, the mucous cells contain less sialic acid and more sulphated mucus (Bullock *et al.* 1976).

Sacciform cells were also counted in the second experiment as it was suggested that the proliferation of these cells may be some form of defense response against ectoparasite infections. It was reported that low sacciform cell counts in brown trout, *Salmo trutta* and Arctic char, *Salvelinus alpinus*, was also related to these fish's susceptibility to parasitic and fungal infections in sexually mature males (Pickering and Fletcher 1987). These cells were AB (-) and PAS (-) but gave highly positive reaction for amino acids and proteins hence Takashima and Hibiya (1995) considered them to be involved in bioactive peptide secretion. Significantly lower sacciform cell concentration was detected only at day 1 p.e. in fish subjected to fluctuating temperature and exposed to fungal spores, and was back to normal for the duration of the experiment. This decline may be an initial response to the temperature changes. Also, exposure to *A. invadans* spores with fluctuating temperature possibly had an additional impact since 3-spot gouramis subjected to such treatments had the lowest sacciform cell counts. Pickering and Fletcher (1987) also reported that the number of sacciform cells is usually inversely correlated with the number of mucous cells in brown trout (*S. trutta*) and Arctic char (*Salvelinus alpinus*). This was confirmed by the relatively higher total mucoud cell counts (Fig. 4.10a) at day 1 p.e. in all treatments.

The inflamed lesions observed in the 2 gouramis subjected to varying temperatures and serial spore exposure at day 24 p.e. can be attributed mainly to

handling, in addition to the temperature stress, which led to the erosion of the anal and caudal fins. In rainbow trout exposed to acidified water and other stressors, invasion of lymphocytes and macrophages occurred due to the damaged epithelial cells in the deeper epidermal layers (Iger *et al.* 1994a; 1994b;1995; Sharples and Evans, 1996). Also, Pickering (1974) reported that there were significantly lower mucous cells in the fins than in the other region of brown trout and Arctic char epidermis, hence these areas may be more sensitive to damage and can be potential entry points for pathogens like *A. invadans* spores.

Taken all together, the temperature treatments and physical stress employed in the trials only produced minor epidermal changes during the 12-day and 24-day experimental periods, possibly affecting only the areas more vulnerable to injury such as the fins at day 24 p.e. hence, there was no *A. invadans* infection induced in three-spot gouramis. It may be pertinent that in a study to determine the effects of *Gyrodactylus colemanensis* and *G. salmonis* on the epidermis of rainbow trout fry, Wells and Cone (1990) reported that significant reduction in the number of mucous cells in the epidermis of the fins was only detected on day 24 post-infection, thus suggesting a longer period of observation may have revealed fungal invasion.

It is apparent that the epidermis and the mucus layer play an important role in the defense mechanism of fish against biological and environmental factors. It has been demonstrated in some species of fish that immunoglobulins are present in skin mucus (Bradshaw *et al.* 1971; Ourth 1980; Itami1993) and that lymphocytes and immunoglobulin-containing plasma cells are present in subepidermal layer (St. Louis-Cormier *et al.* 1984; Peleteiro and Richards 1985). Lysozyme and protease activities were also reported in channel catfish, *Ictalurus punctatus*, and ayu, *Plecoglossus altiveles* (Ourth 1980; Hjelmeland *et al.* 1983; Itami 1993). In winter flounder, *Pleuronectes americanus,* an antimicrobial peptide in the epidermal mucous cells, called pleurocidin, was also reported (Cole *et al.* 1997). Thus, as long as the defense mechanisms provided by the skin remain functional, pathogens would not be able to infect fish cutaneous tissues.

The importance of mucous cell secretions was supported by the results of an experimental infection of rainbow trout (*O. mykiss*) with *Gyrodactylus derjavini* which showed that the number of mucous cells in the fins and skin was weakly correlated with the parasite load at week 1 but this changed into a significant negative correlation as the infection progressed until week 6 when parasite density increased and opted to attach and settle in areas with low numbers of mucous cells like the tail fin and corneal surface (Buchmann and Bresciani 1998). Consequently, Buchmann (1999) proposed a model of the skin immune mechanisms in fish based on the infection of rainbow trout (*O. mykiss*), with the monogenean *G. derjavini*. The model emphasised the significance of mucus in the initial parasite attachment and the subsequent inflammatory and cellular response. It was suggested that the release of cytokines, such as IL-1, from leukocytes following mechanical and chemical injury of the epithelial cells could apparently affect mucous secretions and initiate the inflammatory process during the early stage of the infection while the humoral factors in the mucus were proposed to play a role in the later stages of the infection. Because of the myriad of immune factors in the mucus, the parasites eventually had to attach on a site with less resistance to infection such as the tail and cornea.

Similar defense mechanisms were reported previously by (Wood *et al.* 1986; Wood *et al.* 1988; Willoughby 1989) in brown trout (*S. trutta*) with experimental *Saprolegnia* infection. Combinations of cellular and humoral factors in the mucus and the physical removal of mucus protect the fish from the fungal infection. An *in vitro* study on the affinity of *A. invadans* spores on glass slides coated with sand whiting mucus showed that more spores attached and germinated on mucus-coated slides than on uncoated glass slides (Catap unpub. data). In spite of this affinity, it is highly probable that the immune factors in the mucus, unless compromised, and the continuous shedding of mucus, could likewise prevent the establishment of *A. invadans* on fish skin. Consequently, this should be an important factor to consider when seeking to identify therapeutic or preventive measures against *A. invadans* infection.

Chapter Five*

Studies of the potential of immunomodulators and other prophylactics to ameliorate EUS in susceptible fish

*Experiment on fish oil as diet supplement forms part of a paper published in Fish Pathology, 33:327-335.

5.1 INTRODUCTION

To be fully effective, control measures and prophylactic treatments against EUS, as in other fish diseases, should be based on the pathogenesis of the disease, taking into considerations the interactions of the host, the main pathogen involved and other associated factors. It is thus important that continuous efforts be exerted to identify preventative measures against a multifactorial disease like EUS. In relation to immunoprophylaxis, Thompson *et al.* (1997) reported the non-specificity of the anti-*A. invadans* sera raised in the snakehead, *Channa striata.* In addition, the immunogenicity of a low molecular weight (10 kDa) protein detected in the sera of fish with natural and experimental *A. invadans* infection, was apparently lost when fish were vaccinated with heat-killed fungal zoospores. Hence, the development of a vaccine against *A. invadans* infection still requires further investigations and studies on other modes of prevention and treatment should be undertaken.

Results from experiments which were undertaken to examine the potential of four substances (fish oil, L-cysteine ethyl ester (L-CEE), levamisole and yeast glucan) in ameliorating the effects of *A. invadans* infection during periods of low and/or fluctuating water temperatures, will be presented in this chapter. The effects of oral fish oil supplementation were investigated to determine if such treatment could enhance the inflammatory or cellular response of fish to *A. invadans* by counteracting the immunosuppressive effects of low temperature. As for L-CEE, its mucolytic property could possibly impart protection by facilitating the shedding of epidermal mucus, therefore preventing the attachment of fungal zoospores on the skin of susceptible fish.

Levamisole and yeast glucan are known to stimulate nonspecific immune responses via their target cells, the lymphocytes and macrophages, respectively. The use of these immunostimulatory substances could improve the response of fish during stressful periods, as when temperatures decrease or fluctuate, not only to *A. invadans* invasion but also to secondary infections, which occur commonly in EUS lesions. Different cellular and humoral non-specific parameters were

measured in experimentally-infected sand whiting to determine the efficacy of these two substances against *A. invadans* infection.

5.2 MATERIALS AND METHODS

5.2.1 Diet supplementation with fish oil

This experiment was undertaken simultaneously with Experiment I which was presented in Chapter Three using the second group of sand whiting fed with a high fish oil diet during the acclimation period. A high fish oil diet, prepared by adding 10 g refined fish oil per 100 g of Gibson's salmon pellet (2.0 mm Ø, 10% initial oil content), was fed to sand whiting for 42 days (6 weeks). Fingerlings (15 x 12 tanks) were divided into 4 treatment groups: a) negative (-) control, injected with PBS, no temperature manipulations; b) positive (+) control, injected with *A. invadans* zoospores, no temperature manipulations; c) sand whiting subjected to gradual temperature decrease and then injected with *A. invadans* zoospores and d) sand whiting subjected to rapid temperature drop and then injected with *A. invadans* zoospores. Each treatment was done in three replicates.

Husbandry conditions during acclimation and experimental periods, fungal inoculation, temperature manipulations, sampling and histopathological methods were as described in Experiment I of Chapter Three.

5.2.2 Diet supplementation with L-CEE

This experiment was undertaken simultaneously with Experiment II which was also presented in Chapter Three. For the L-CEE treatment, fish were fed with diet supplemented with the mucolytic agent for three days (10 mg L-CEE/kg of biomass/day) prior to the gradual temperature drop and addition of fungal mats. The required dosage of L-CEE was added by coating the dry salmon pellet with a mixture of 10% gelatine and L-CEE (ICN Biomedical Research Products).

The mucolytic agent, L-CEE had been reported as an anti-phytoplankton therapy in coho salmon, *Oncorhynchus kisutch*, against *Chaetoceros concavicornis*. This phytoplankton could induce excessive mucus production in the gills and could accumulate in between secondary gill lamellae leading to fish suffocation. The use of L-CEE lead to reduced mucus production, hence mucus and phytoplankton did not accumulate in the gill lamellae (Yang and Albright 1994). For this experiment, this substance was used as a therapeutic agent to induce shedding of mucus to prevent the attachment of *A. invadans* on the skin of sand whiting.

Husbandry conditions during the acclimation and experimental periods, fungal mats and zoospore treatments, temperature manipulations, sampling and histopathological methods were as described in Experiment II of Chapter Three.

5.2.3 LEVAMISOLE AND YEAST GLUCAN TRIALS. Two trials were undertaken to investigate the effects of levamisole and yeast glucan on some non-specific immune responses in sand whiting injected with *A. invadans* zoospores and to compare the effects of the two immunostimulants in fish held at 26 °C and in fish subjected to gradual drop in temperature and then held at 17 °C.

5.2.3.1 Fish and husbandry conditions

Experiment I.A Fish held at 26 °C. Sand whiting (Bribie Island Aquaculture Research Centre, DPI Queensland) reared at the Aquatic Centre of the School of Aquaculture (n=102; mean wt. = 68.2 ± 19.0 g) were randomly distributed and acclimated for 14 days in 8 x 250-L static polypropylene tanks with immersion heaters and continuous aerators. Fish were fed with Gibson's dry salmon pellets (2.5 mm Ø) at 1.0% total body weight per day. One-third to one-half of the water was changed every two days and water quality was monitored daily. Full-strength seawater was mixed with municipal water in a 1000-L Relm tank with continuous aeration and similar temperature as in the experimental tanks. Salinity ranged from 4.0-8.0 ‰ (mean=6.1 ± 1.5 ‰), temperature from 25.5-28.2 °C (mean = 26.2 ± 0.5 °C) and DO from 2.4- 7.1 mgL^{-1} (mean = 6.1 ± 0.91 mgL^{-1}) during the acclimation period. Level of NH_3-N was from <0.1-0.5 mgL^{-1}.

Experiment I.B Fish subjected to gradual temperature drop. Sand whiting (n=96; mean wt.=33.4 ± 5.5 g) were acclimated in 8 x 250-L static polypropylene tanks for 7 days prior to any temperature manipulations. Water temperature during the acclimation period was maintained through the use of immersion heaters. The same feed, feeding rate and water exchange regimes used during the acclimation period in the first experiment were utilised for this trial. Salinity ranged from 5-7.5 ‰ (mean= 5.7 ± 0.86 ‰), temperature from 25.4-26.6 °C (mean=25.9 ± 0.3 °C), DO from 5.5-7.4 mgL^{-1} (mean= 6.7 ± 0.5 mgL^{-1}), NH_3-H from 0.1-0.25 mgL^{-1} and pH from 6.4-7.0.

5.2.3.2 Experimental design

The same experimental design was employed for the two experiments. After acclimation, the 8 tanks of fish for each experiment were assigned to 4 different experimental treatments: 1) negative (-) control group, injected with PBS; 2) positive (+) control group, injected with *A. invadans* zoospores; 3) levamisole-treated group, fed with levamisole-supplemented diet and then injected with *A. invadans* zoospores; and 4) yeast glucan-treated group, fed with yeast glucan-supplemented feed and then injected with *A. invadans* zoopores. Each treatment was done in two replicates and temperature was maintained at ~ 26 °C during the entire experimental period. However, for the second experiment, water temperature was gradually dropped from 26 °C to 17 °C within 7 days before the fish were inoculated with either PBS or fungal zoospores. The temperature was then maintained at 17 °C until tissue samples were obtained for analyses at day 8 p.i.

The (-) control and (+) control groups were continuously fed with unsupplemented dry salmon pellets prior to any inoculation. The levamisole-treated fish were fed with dry salmon pellet supplemented with levamisole (L9756, Sigma, USA, $C_{11}H_{12}N_2S.HCl$) at 5mg/kg body weight/day every 3 days for 15 days. This dosage was based on the work of Siwicki (1989) who reported the optimal stimulatory dose of levamisole as a feed supplement from 3-8 mg/kg of fish body weight. Yeast glucan (Aquaguard, Aquatic Diagnostic Services,

Australia) was incorporated with the salmon pellet at 1.0 g Aquaguard/kg dry feed and fed daily for three weeks, based on the recommended dosage by Aquatic Diagnostic Services. Feeding rate was 0.8% total body weight for all treatments.

In Experiment I.A, fish were allowed to recover for one week after oral feeding of levamisole- or glucan-supplemented diets before zoospore inoculation, while in Experiment I.B, water temperature was slowly decreased for one week after the oral immunostimulation treatments and fish were then injected with zoospores.

5.2.3.3 Production of *A. invadans* zoospores and preparation of inocula

Methods to produce fungal mats and zoospores were as described in Chapter Two (Section 2.2.2). For the two experiments, a zoospore suspension of ~1.5 x 10^3 sporesmL^{-1} was prepared by diluting the primary spore suspension with autoclaved pond water and was used as inocula.

5.2.3.4 Fish inoculation

Fish were anesthetised with 60 mgL^{-1} benzocaine before spore inoculation. Fish in 2 tanks were each injected with 0.1 ml phosphate-buffered saline (PBS), as (-) control (Group I), in the dorsal muscle, just anterior to the base of the dorsal fin. Fish in tanks as (+) control (Group II), levamisole-treated (Group III) and glucan-treated (Group IV) were each injected with 0.1 ml of ~1.5 x 10^3 spores/ml of. *A invadans* spore suspension (about 150 spores). After inoculation, fish were revived in well-aerated, clean water and transferred to their respective treatment tanks.

5.2.3.5 Experimental conditions and sampling procedures

During the 8-day experimental period, water exchange was done every two days, with one-half of the water in each tank replaced with municipal water mixed with full-strength seawater (as in acclimation period). Salinity, temperature and DO levels were monitored daily while NH_3-N level was determined once during the experiment. The pH level was not measured during these trials since the previous

experiments showed that pH levels did not fluctuate markedly which could be due to regular water exchange. The fish were fed daily with dry salmon pellet for the duration of the trial. For the second experiment, feeding rate was decreased to 0.5% of the total body weight when the water temperature reached 17 °C. Fish were observed daily for gross signs of infection or any mortality. At day 8 p.i., fish were euthanased with 100 mgL^{-1} benzocaine to collect blood, anterior kidney and muscle tissues samples.

5.2.3.6 Collection of blood, anterior kidneys and muscle tissue

Blood was collected from each fish via the caudal vein using a 2.0 ml syringe and needle (gauge 23) flushed with heparinized saline (50 units/ml PBS). Collected blood was transferred to blood sample tubes with dipotassium EDTA (Sarstedt, Australia). Using a micropipettor, an aliquot of the blood sample from each fish was placed in a microcapillary tube and sealed at one end with Cha-seal (Chase Instruments Corp. Cat. no.510). Blood smears were also prepared for each blood sample. The remaining blood in each sample tube was kept on ice while other tissue sample were dissected out and collected.

Before dissecting out the head kidneys, the fish were bled by cutting the caudal fin and were sprayed with 70% ethyl alcohol. The collected kidneys were immediately placed in sterile Petri dishes with cold supplemented fish physiological saline (FPS pH 7.2, NaCl=6.44 g, KCl=11 mg, $CaCl_2$=22 mg, $MgSO_4$=12 mg, KH_2PO_4=7 mg, $NaHCO_3$=10 mg dissolved in 1L distilled water, autoclaved and 10 g glucose added prior to vacuum filtration). Tissue samples were stored at 4 °C overnight when isolation/separation of phagocytic cells could not be performed on the same day (Judith Zelikoff, pers. comm.).

Muscle and skin at the inoculation site of each fish was cut and immediately fixed in cold 10% phosphate-buffered neutral formalin for histopathological examination to confirm *A. invadans* infection.

5.2.3.7 Haematological indices

Haematocrit values

The sealed microcapillary tubes with blood were centrifuged (micro-haematocrit centrifuge, Gelman-Hawkesley Ltd. England) for 4 min at 1100 rpm. After centrifugation, haematocrit values were determined by measuring the column length of packed RBC and length of total blood in the microcapillary tubes using a ruler under a dissecting microscope. The haematocrit values were obtained by calculating the packed RBC as percentage of the total blood in the microhaematocrit tubes.

Differential white blood counts

The air-dried blood smears were fixed in 95% methanol for 2 min and stained with eosin (12 dips, Quick Dip solution 1, Histo Labs Pty. Ltd.) and Giemsa (8 dips, Quick Dip solution 2), rinsed in tap water, blotted dry and air-dried. When completely dried, 2-4 drops of DPX mountant was placed on each slide and covered with a glass coverslip. The mounted slides were examined microscopically (Olympus BH-2) using the oil immersion objective. A total of 100 WBC/smear were counted and values were expressed as percentage of each cell type (% cell type = total no.of each cell type counted/100 cells counted x 100%).

5.2.3.8 Nonspecific immune parameters

Phagocytic activity

Head kidney cell isolation and preparation

The method used was based on Zelikoff *et al.* (1996) with some modifications. Head kidneys in cold FPS were pooled (with 2-3 head kidneys/pool; 3 to 5 pools per replicate) and passed through sterile wire mesh/sieve in a Petri dish with 3 mL cold, L-15 medium (Leibovitz medium with 200 mM of L-glutamine, Sigma) supplemented with 0.5% sand whiting plasma, 1% penicillin/streptomycin

(Sigma, USA cat. no.P 0781). The cell suspension was then passed though a loosely packed sterile glass wool-plastic syringe column to remove tissue/cellular debris and red blood cells. The cells were recovered in a 15 mL sterile polypropylene centrifuge tube and layered carefully over Histopaque 1077 solution (Sigma, cat. no.1077-1) and centrifuged (BHG Roto-Uni II, Germany) for 15 min (400 x g) at room temperature.

After centrifugation, the white blood cell band was removed with a sterile Pasteur pipette and washed twice with 3 mL supplemented L-15 medium for 10 min/wash at 1500 rpm. After the final wash, the supernatant was removed and the pelleted cells were resuspended in 3 mL supplemented FPS. Cell number was determined by haemocytometer counting and the cell viability by trypan blue exclusion method.

Preparation of congo red-coated yeast cells

Yeast cells were suspended in PBS (pH 7.2) adjusted to a concentration of 10^8 cells/mL. Congo red (5 mL, 0.8%) was added to 10 mL of the yeast suspension and then autoclaved. The yeast cells were washed in sterile PBS for at least 4 times (3 min, 300 rpm) to remove excess dye and resuspended in the same volume of PBS to maintain required yeast cell concentration.

In vitro *phagocytosis assay*

Resuspended cells in supplemented FPS were adjusted to 5 x 10^6 cells/mL in supplemented L-15 medium to prepare the cell suspensions. A mixture of 100 µL of the cell suspension and 50 µL of the Congo red-coated yeast cells was placed in each well (5 wells/sample) of a 96-well flat-bottomed plate (Greiner Labortechnik) and incubated for 2 hours at 26 °C in a humidified environment. Samples from the second experiment were incubated at 17 °C.

After incubation, 20 µL of the mixture in one well was pipetted on a glass slide to prepare a smear. The smears were air-dried, fixed in 95% ethyl alcohol and stained using the Quick Dip solutions 1 and 2. After drying, the slides were mounted with DPX and glass cover slips.

At 1000x magnification, 300 cells were counted/smear to determine the phagocytic activity which is equal to the [total number of cells containing one or more yeast cells / total number of cells counted] x 100. Cells were considered positive if they contain yeast or were actively phagocytosing a yeast particle (50% inside).

Lysozyme activity

Blood samples in the blood sample tubes were pooled (2-4 samples/pool; 3 pools per replicate), transferred to 1.5 mL Eppendorf tubes and centrifuged (MSE Microcentaur) at 13000 rpm for 5 min. After centrifugation, plasma was collected in Eppendorf tubes and stored at –20 °C until the assays (lysozyme and antiprotease activity) were performed.

Lysozyme activity was determined by a microtitre plate method described in Thompson *et al.* (1994) which measures the lysis of a suspension of *Micrococcus lysodeikticus* (75 mg 100 mL^{-1} of 0.1 M phosphate/ citrate buffer with 0.09% NaCl, pH 5.8). The optimum lysozyme activity of sand whiting plasma using this method was obtained at pH 5.8, hence the buffer used for all the assays was at pH 5.8. One hundred and seventy five µL of the bacterial suspension was added to 25 µL of each plasma sample, and to hen egg white lysozyme (Sigma, cat. no. L-6876) standards (0-50 µg mL^{-1} phosphate/citrate buffer, pH 6.0 optimum) in flat bottomed, 96-well plates, in triplicate wells per sample. The rate of lysis was determined against a *M. lysodeikticus* (Sigma, cat. no. M-3770) blank during a 5 min kinetic run (15 sec intervals) at 450 nm on a multiscan spectrophotometer (Titertek Multiskan® MCC/340) at 25 °C. Lysozyme activity was calculated from the standard curve, and expressed as µg lysozyme mL^{-1} plasma.

Antiprotease activity

The same plasma samples were used for this assay. Antiprotease activity was determined using the method described in Thompson *et al.* (1994) which measures the anti-trypsin activity of plasma. Samples were serially diluted in PBS, pH 7.2, in round bottom, 96-well plates. Five µL aliquots were transferred

to a flat bottomed, 96-well plate , in duplicate, and 15 µL trypsin (Sigma, cat. no. T-8003; at 100 µg mL^{-1} PBS) was added per well, mixed and incubated for 5 min at 25 °C. Two hundred µL of BAPNA (Nα-benzoyl-dl-arginine p-nitroanilide, Sigma, B-4875) substrate solution (10.4 mg BAPNA per 2 mL of DMF (dimethylformamide, Sigma D-8654, in 20 ml of 0.01M tris/$CaCl_2$ buffer, pH 7.8) was added to all wells and antitrypsin activity was determined during a 15 min kinetic run (30 sec intervals) in a multiscan spectrophotometer (Titertek Multiskan® MCC/340) at 405 nm against a PBS/BAPNA blank. For each plasma sample, a graph of Vmax rates (mOD/min) against plasma volume was plotted to obtain µL required to give 50% inhibition. Data were expressed as no. of trypsin units inhibited per µL plasma using the following formula:

$$\frac{\text{Vmax value x 15 x 0.001 x 80.8 x 0.50}}{\text{µL obtained from curve}} = \frac{\text{no. of trypsin units inhibited}}{\text{µL of plasma}}$$

5.2.3.9 Histopathology

Fixed tissue samples were decalcified, processed and embedded in paraffin wax. Sections (5 µm) were stained using the periodic acid-Schiff method. Sections were examined using an Olympus BH-2 light microscope to confirm *A. invadans* infection.

5.2.4 YEAST GLUCAN TRIALS. Additional two experiments, with few modifications on the experimental design, were undertaken to further investigate the effects of orally-supplemented yeast glucan on *A. invadans*-infected sand whiting, either maintained at 26 °C or subjected to gradual temperature decrease (from 26 °C to 19 °C). For these trials, the production of extracellular superoxide anion (a measure of the respiratory burst) and the alternative complement activity were measured as indices of non-specific immune responses.

5.2.4.1 Fish and husbandry conditions

Experiment II.A Fish held at 26 °C. Sand whiting (163.0 ± 24.0 g mean wt., Fig. 5.1) were randomly distributed in 250 L polypropylene, static tanks (8 fish x 6 tanks) with continuous aeration and immersion heaters to maintain the water temperature. The fish were acclimated for one week with daily feeding at 2% total body weight (Gibson's 3.0 mm Ø dry salmon pellet) and one-third of the water replaced every two days with a mixture of municipal water and full-strength seawater to achieve the required salinity. Salinity, temperature and DO were monitored regularly, while NH_3-N was measured once during the acclimation period. Salinity was maintained at 5 ‰, temperature ranged from 25.0-27.0 °C (mean=25.8 ± 0.8 °C) and DO ranged from 3.2-3.9 mgL^{-1} (mean= 3.5 ± 0.3 mg L^{-1}) while NH_3-N was not detectable with Aquasonic® measuring kit.

Experiment II.B Fish subjected to gradual temperature drop. Sand whiting (123.2 ± 23.7 g mean wt.) were acclimated in 250 L static tanks (4 fish x 6 tanks) with continuous aeration and immersion heaters. Similar feeding and water exchange regimes used in Experiment II.A were employed for this experiment. Salinity was also maintained at 5 ‰, temperature ranged from 25.0-27.0 °C (mean=25.9 ± 0.5 °C), DO ranged from 4.1-5.8 mgL^{-1} (mean=5.2 ± 0.4 mgL^{-1}), pH from 6.4-6.6 while NO_2^- was <0.1 mgL^{-1} and NH_3-N was not detectable.

5.2.4.2 Experimental design

After the acclimation period, the six tanks of fish were randomly allocated for the 3 experimental treatments, with two replicate tanks per treatment: 1) negative (-) control, fish injected with autoclaved pond water only; 2) positive (+) control, fish injected with *A. invadans* zoospores; and 3) glucan-treated fish, fish fed with yeast glucan supplemented diet and then injected with *A. invadans* zoospores. In Experiment II.A, temperature was maintained at ~26 °C during the 8-day experimental period. The (-) and (+) control fish were fed continuously fed with Gibson's 3.0 mm Ø dry salmon pellet. Yeast glucan was incorporated with the salmon pellet at 1.0 g Aquaguard/kg dry feed and the fish were fed daily for three weeks and then injected with *A. invadans* zoospores. Feeding rate was at 1.5%

total body weight. For Experiment II.B, the same treatments were used as in Experiment II.A. The fish were also fed with glucan-supplemented feed at the same feeding rate daily for three weeks. The water temperature was dropped from 26 °C to 19 °C over a period of 7 days (3rd week of glucan feeding), and then fish were injected with *A. invadans* zoospores.

5.2.4.3 Fish inoculation

Aphanomyces invadans mats were grown and allowed to sporulate using the methods described in Chapter Two (Section 2.2.2). For the two experiments, a zoospore suspension of ~5.0 x 10^3 spores/ml of APW was prepared and used as inocula. The fish were anesthetised with 60 mgL^{-1} benzocaine before inoculation. The (-) control fish each received an intramuscular injection of 0.5 ml APW in the dorsal muscle, just anterior to the base of the dorsal fin. Fish in the (+) control and the glucan-treatment groups were each injected with 0.5 ml of the *A invadans* spore suspension (about ~2,500 spores). After inoculation, fish were revived in well-aerated, clean water and transferred to their respective treatment tanks. The same inoculation procedures were used in Experiment II.B.

5.2.4.4 Experimental conditions and sampling

During the 8-day experimental period, water exchange was performed every two days, with one-third to one-half of the water in each tank replaced with municipal water mixed with full-strength seawater (as in acclimation period). Salinity, temperature and DO levels were monitored regularly. The fish were fed daily with the dry salmon pellet for the duration of the trials. For the second experiment, feeding rate was decreased to 1.0 % of the total body weight during the experiment when the water temperature was 19 °C. Fish were observed daily for any gross signs of infection and mortality. At day 8 p.i., fish were euthanased with an overdose (~200 mgL^{-1}) of benzocaine to collect blood, anterior kidney and muscle tissues samples.

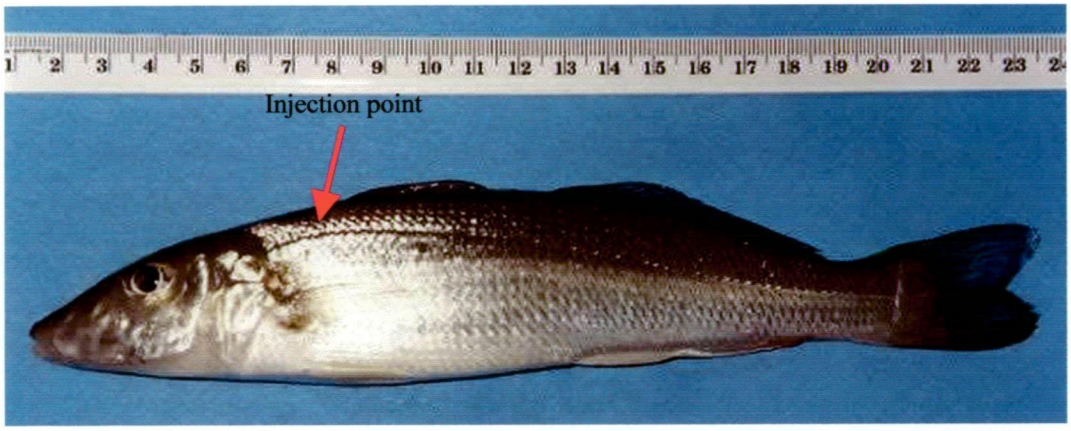


Fig. 5.1 Experimental sand whiting injected with *A. invadans* spores.

5.2.4.5 Collection of blood, anterior kidneys and muscle tissue

Blood was collected from each fish through the caudal vein using a 3.0 ml syringe and needle (gauge 23) flushed with heparinized saline (50 units/ml PBS). Collected blood, which will be used to obtain serum for the alternative complement activity assay, was transferred to 1.5 mL Eppendorf centrifuge tubes and was kept on ice while other tissue samples were dissected out and collected. Before dissecting out the head kidneys, the fish were bled by cutting the caudal fin and were sprayed with 70% ethyl alcohol. The collected kidneys were immediately placed in sterile Petri dishes with cold supplemented FPS and kept at 4 °C until the isolation of macrophages for the respiratory burst assay was performed. Muscle and skin at the inoculation site of each fish was cut and immediately fixed in cold 10% phosphate-buffered neutral formalin for histopathology examination.

5.2.4.6 Nonspecific immune parameters

Respiratory burst assay

Head kidney cell isolation and preparation

Head kidneys in cold FPS were passed through sterile wire mesh/sieve in a Petri dish with 3 mL cold, L-15 medium (Leibovitz medium with 200 mM of L-glutamine, Sigma) supplemented with 0.5% sand whiting plasma, 1% penicillin/streptomycin (Sigma, USA cat. no. P-0781). The cell suspensions were then passed though a loosely packed sterile glass wool-plastic syringe column to remove tissue/cellular debris and red blood cells. The cells were recovered in a 15 mL sterile polypropylene centrifuge tubes and layered carefully over a Percoll (Pharmacia Biotech, Sweden) discontinuous density gradient (1.035/1.065-optimised for sand whiting head kidney cells) and centrifuged (BHG Roto-Uni II, Germany) for 15 min (400 x g) at room temperature to obtain a macrophage-enriched layer. After centrifugation, the white blood cell band was removed with a sterile Pasteur pipette and washed twice with 3 mL supplemented L-15 medium for 10 min/wash at 1500 rpm. After the final wash, the supernatant was removed and the pelleted cells were resuspended in 3 mL supplemented FPS. Cell number

and viability were determined by haemocytometer counting and trypan blue exclusion method (95-98% viability), respectively. The cell suspension was adjusted to 4 x 10^6 cells/ml supplemented L-15 medium and kept on ice until the reaction tubes for the respiratory burst assays were prepared.

Extracellular superoxide anion production

The method used was based on Zelikoff *et al.* (1996) which measures the reduction of ferricytochrome C (Sigma, C-7752, 4 mg mL^{-1} in supplemented FPS as stock solution) in cells stimulated by phorbol 12-myristate 13-acetate or PMA (Sigma, P-8139) in the presence of superoxide dismutase or SOD (Sigma, S-2515, 300 μg mL^{-1} in Hank's balanced salt solution or HBSS as stock solution). Stock solution of PMA prepared in dimethylsulfoxide (DMSO, Sigma D-5879) at a concentration of 1 mg mL^{-1} was diluted with HBSS to obtain a working solution of 10 μg mL^{-1}. For each sample of cell suspension, reaction mixtures used to measure extracellular O_2^- production were prepared in Eppendorf tubes as follows (Table 5.1):

Table 5.1 Reaction mixtures used in the extracellular O_2^- production .

	Blank	**Tube 1 Unstim w/o SOD**	**Tube 2 Unstim w/ SOD**	**Tube 3 Stim w/o SOD**	**Tube 4 Stim w/ SOD**
Ferricytochrome C	500 μL	500 μL	500 μL	500 μL	500 μL
Cell suspension (4 x 10^6 cells mL^{-1})		250 μL	250 μL	250 μL	250 μL
SOD	125 μL		125 μL		125 μL
PMA (added last)	20 μL			20 μL	20 μL
FPS	355 μL	250 μL	125 μL	230 μL	105 μL
Total volume/tube	1000 μL	1000 μL	1000 μL	1000 μL	1000 μL

Final concentration of ferricytochrome C was 2 mg mL^{-1}, SOD was at 37.5 μg mL^{-1} while PMA was at 0.2 μg mL^{-1}. This PMA concentration was determined to be the optimum for sand whiting head kidney macrophage.

After the reaction tubes were prepared, each tube was vortexed and 200 µL aliquots (2 x 10^5 cells/well) were placed into individual wells of a 96-well microtitre plate and the absorbance measured at 550 nm using a multiscan spectrophotometer (Titertek®Plus MS 212) for up to 150 min. Timepoints used to measure the respiratory burst were 0, 15, 30, 45, 60, 90, 120 and 150 min.; plates were incubated in a humid environment at 26 °C between readings for the samples from Experiment II.A while samples from Experiment II.B were incubated at 19 °C between readings.

Data were expressed as nmol O_2^-/2 x 10^5 cells/unit time which were calculated by multiplying the change in absorbance (mean of non-SOD wells minus mean of SOD wells) by 15.87. For these experiments, data taken during the 60 min and 120 min timepoints were used to compare different treatment groups.

Alternative complement activity

Blood samples in 1.5 mL centrifuge tubes were allowed to clot for at least 3 hrs and then centrifuged (MSE Microcentaur) at 13000 rpm for 5 min. After centrifugation, sera were collected in Eppendorf tubes and stored at –20 °C until the assays were performed.

The method used for the alternative complement pathway was described by Yano (1992) which measures the amount of serum required to lyse a given number of rabbit eythrocytes (RaRBC) in the presence of EGTA and Mg^{2+} to block the classical complement pathway. The optimal pH (7.5) value for the buffer and serum dilution (1/20) were determined for sand whiting serum before the main assays were performed.

RaRBC in Alsever's solution (Veterinary Services Division, IMVS, South Australia) were washed 3 times in 0.01 M EGTA-Mg-gelatine veronal (sodium barbiturate) buffer and cell concentration was adjusted to 2 x 10^8 cells mL^{-1} of the buffer. Different volumes of the diluted sample sera (1/20) were prepared and

placed in test tubes (1.0 cm x 7.5 cm, Kartell Dispolab) with the buffer and RaRBC suspension. The reaction tubes for the assay are shown in Table 5.2.

Table 5.2 Reaction mixtures used for the alternative complement activity.

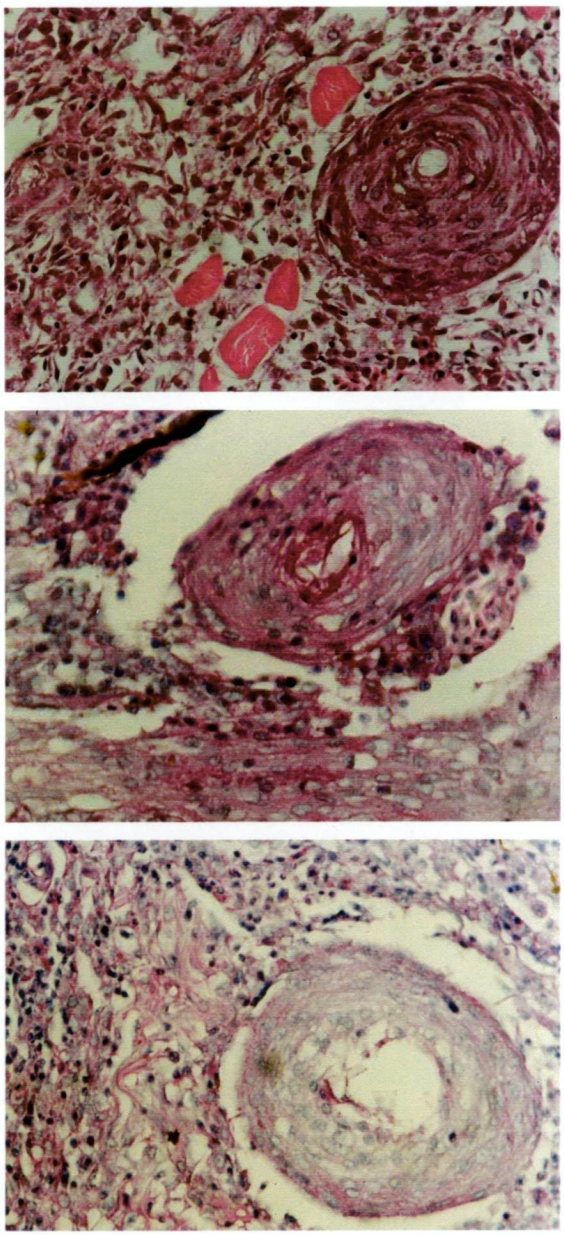
Tube no.	1	2	3	4	5	Cell blank	100% lysis
EGTA-Mg-GVB (ml)	-	0.050	0.100	0.150	0.200	0.250	-
Serum (dil: 1/21) (ml)	0.250	0.200	0.150	0.100	0.050	-	-
Distilled water (ml)	-	-	-	-	-	-	3.40
RaRBC ($2x10^8$/ml) (ml)	0.100	0.100	0.100	0.100	0.100	0.100	0.100

All the tubes were incubated for 90 min at 26 °C (sera from Expt. I) and 19 °C (sera from Expt. II). After incubation, 3.15 mL of physiological saline was added to all tubes (except the 100% lysis control) to make the volume up to 3.50 mL and were centrifuged (Econospin, Sorvall Instruments) at 1600 x g for 5 min. The supernatants were then pipetted into cuvettes (Kartell Dispolab) and the absorbance read at 414 nm (Shimadzu UV-1200). Absorbance readings were corrected by subtracting the value of the cell blank and the degree of hemolysis (y) was calculated by dividing each corrected absorbance value by the corrected absorbance of the 100% hemolysis control. The values were then plotted against the mL of (x) serum (best fit line) to obtain amount of serum which produced 50% hemolysis (K). The ACH_{50} value was calculated using the following formula:

$$ACH_{50}\ (unit/mL) = 1/K \times (\text{reciprocal of the serum dilution}) \times ½$$

5.2.4.7 Histopathology

Fixed tissue samples were decalcified, processed and embedded in paraffin wax. Sections (5 μm) were stained using the periodic acid-Schiff method and examined quantitatively by classifying the fungal granulomas into 3 stages based on their morphology (Fig.5.2). The differential granuloma counts were expressed as percentages of the total counts (50 for Expt. II.A , at least 20 for Expt II.B).



Stage I. Epithelioid and fibroblasts cells form an envelope around an active (round, full Ø seen) fungal hypha.

Stage II. Epithelioid cells reduced in number and fibroblasts appear elongated and flat and the fungal hypha appear collapsed.

Stage III. Lysed fungal hypha; only debris of fungal wall seen in the centre of the granuloma; peripheral fibroblasts in process of maturing fibrocytes.

Fig. 5.2 Photomicrographs of mycotic granulomas showing the 3 stages used as basis for the differential granuloma counts (PAS stain).

5.2.5 Statistical analyses

Two-way ANOVA (full factorial) was performed to analyse the granuloma counts and percentage of cellular infiltration from fish fed with a high fish oil diet. Prior to the ANOVA, normality and homogeneity of variance (Bartlett's) tests were performed on the data sets. The granuloma counts were square root-transformed while the percentage of cellular infiltration data were arcsine square-root transformed. Tukey-Kramer HSD test was used to compare multiple means at $P<0.05$. Data from fish fed with the high and low oil diets were also analysed and compared to evaluate the effects of fish oil supplementation on *A. invadans*-infected sand whiting exposed to either 26 °C or 17 °C. Full factorial 3-way ANOVA was performed with fish oil levels, temperature and sampling days and their interactions as factors.

For the L-CEE treatment, epidermal thickness measurements and mucous cell counts were analysed with the data from (+) control and cohabitation treatments presented in Chapter Three. Full factorial 3-way ANOVA was performed with temperature, infection method and sampling days and their interactions as factors. Data were arcsine square root-transformed prior to the ANOVA and multiple comparison of means was performed using the Tukey-Kramer HSD test at $P<0.05$.

One-way ANOVA was used to analyse the data from each Experiments I.A and I.B. Lymphocyte, monocyte and thrombocytes percentages, lysozyme and antiprotease levels were arcsine square root-transformed. Two-way ANOVA was also used, with temperature (26 °C and gradual drop) and treatments [(-) control, (+) control, glucan and levamisole groups] and their interaction as factors, to determine the effects of temperature on the haematological and non-specific immune parameters. The treatment means were compared using Tukey-Kramer HSD test. For Experiments II.A and II.B, the histopathology quantitative data and the alternative complement activity were also arcsine square-root transformed before the two-way ANOVA. There was no significant difference in the extracellular O_2^- production between replicates, thus the data were pooled for each treatment.

5.3 RESULTS

5.3.1 Effects of fish oil as feed supplement

5.3.1.1 Water quality in the experimental tanks

Table 5.3 shows the range of values for the different water quality variables measured during the experimental period.

Table 5.3 Range of values for each water quality variable measured during the experiment.

	Salinity (ppt)	Dissolved Oxygen (mg/l)	Temperature (°C)	NH_3-N (mgL^{-1})	pH	Alkalinity (mgL^{-1} $CaCO_3$)
High Fish Oil Diet						
1. (-) Control	8-11	6.0-8.1	25.2-26.6	<0.1-0.5	7.2-7.4	75
2. (+) Control	7-11	5.2-8.3	24.7-26.3	<0.1-0.5	7.2-7.4	75
3. Gradual drop	7-13	7.1-8.6	17.1-17.6	<0.1-0.5	7.2-7.4	75
4. Rapid drop	7-11	6.8-8.7	17.2-17.5	<0.1-0.5	7.2-7.4	75

5.3.1.2 Fish mortality and gross observations

As in the experiment which involved sand whiting fed with a low fish oil diet, mortality among fish fed with a high fish oil diet was minimal in the different treatment tanks. Table 5.4 shows the fish mortality incurred during the experimental period for both groups of fish. In treatments subjected to decreasing water temperature, mortality associated with *A. invadans* infection only occurred in fish fed with high fish oil diet subjected to rapid drop in temperature. Gross observations were similar to those seen in fish fed with low fish oil diet. Fish

maintained at 26 °C [(+) control] still swam and ate actively while fish subjected to low temperatures were sluggish and had reduced appetite when external signs of *A. invadans* were evident.

Table 5.4 Sand whiting mortality during the experimental period.

Low fish oil	**(-) Control**	**(+) Control**	**Gradual drop**	**Rapid drop**
Tank 1	0/15	1/15	0/14	0/15
Tank 2	0/15	1/15	0/13	0/15
Tank 3	2/15	1/13	0/14	0/14
High fish oil				
Tank 1	0/15	1/15	0/11	1/13
Tank 2	2/13	2/14	0/12	2/13
Tank 3	1/12	1/15	0/11	0/12

5.3.1.3 Histopathology

Qualitative examination

Qualitative examinations did not reveal marked difference between fish fed with low and high fish oil diets. In Section 3.3.3. of Chapter 3, the pathological changes in PBS- injected fish were described while those of the *A. invadans* zoospore-injected fish were shown in Table 3.4. *Aphanomyces invadans*-infected fish fed with high fish oil diet did not show remarkable inflammatory and granulomatous response when subjected to either rapid or gradual temperature

drop. These fish likewise exhibited delayed granuloma formation, extensive myonecrosis due to prolonged and minimal cellular infiltration and delayed resolution of the lesions. Figures 5.3, 5.4, 5.5,5.6 and 5.7 show the qualitative changes exhibited by infected fish held at 26 °C and those subjected to low temperature.

Quantitative examination

Mycotic granuloma counts

Significant differences were detected between the treatments due to temperature (P=<0.001), sampling days/time (P=<0.001) and the interaction of temperature and sampling days (P=<0.001). Figure 5.8 shows the granuloma counts in sand whiting fed with a high fish oil diet with experimental EUS subjected to different temperatures. Significantly higher granuloma counts were counted in fish held at 26 °C (positive control fish) than in fish subjected to either rapid or gradual temperature drop and then maintained at 17 °C. From day 8 to day 16 p.i., fish kept at low temperature had significantly lower granuloma counts compared with the respective (+) control fish. At day 18 p.i., no significant difference was detected among the 3 treatments since at this period, fish kept at 26 °C had started the healing process manifested by regenerated muscle fibres and predominantly fungal debris in the lesion area. There was also a delay of 6-8 days in the formation of granulomas in fish held at 17 °C since distinct granulomas were initially observed at day 10 p.i. while fish held at 26 °C showed clear mycotic granulomas as early as day 4 p.i.

Percent cellular infiltration

Statistical analyses also showed significant differences between treatments due to temperature (P=<0.001), sampling day (P=<0.001) and the interaction of the two factors (P=<0.001). Figure 5.9 shows the percentage of cellular infiltration measured from sand whiting fed with a high fish oil diet with experimental EUS subjected to different temperatures.

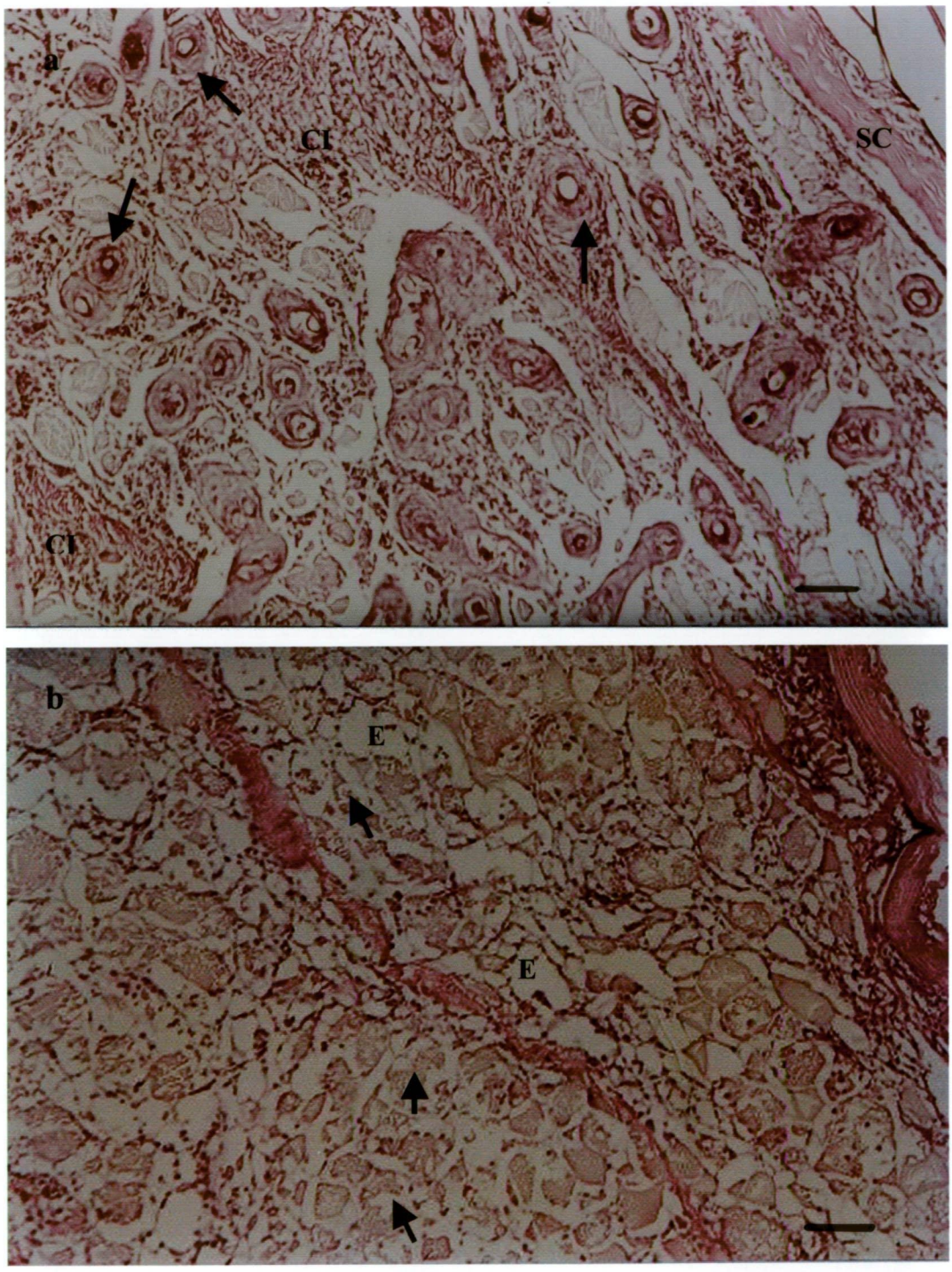


Figure 5.3 Photomicrographs of sand whiting at day 8 p.i. **a) high temp**: distinct mycotic granulomas (arrows) with marked cellular inflammation (CI) in the muscle area, the *stratum compactum* was necrotic (SC); **b)rapid temp drop**: myonecrosis (arrows) evident with oedematous (E) response but no mycotic granulomas present. (PAS stain, bar=100 μm)

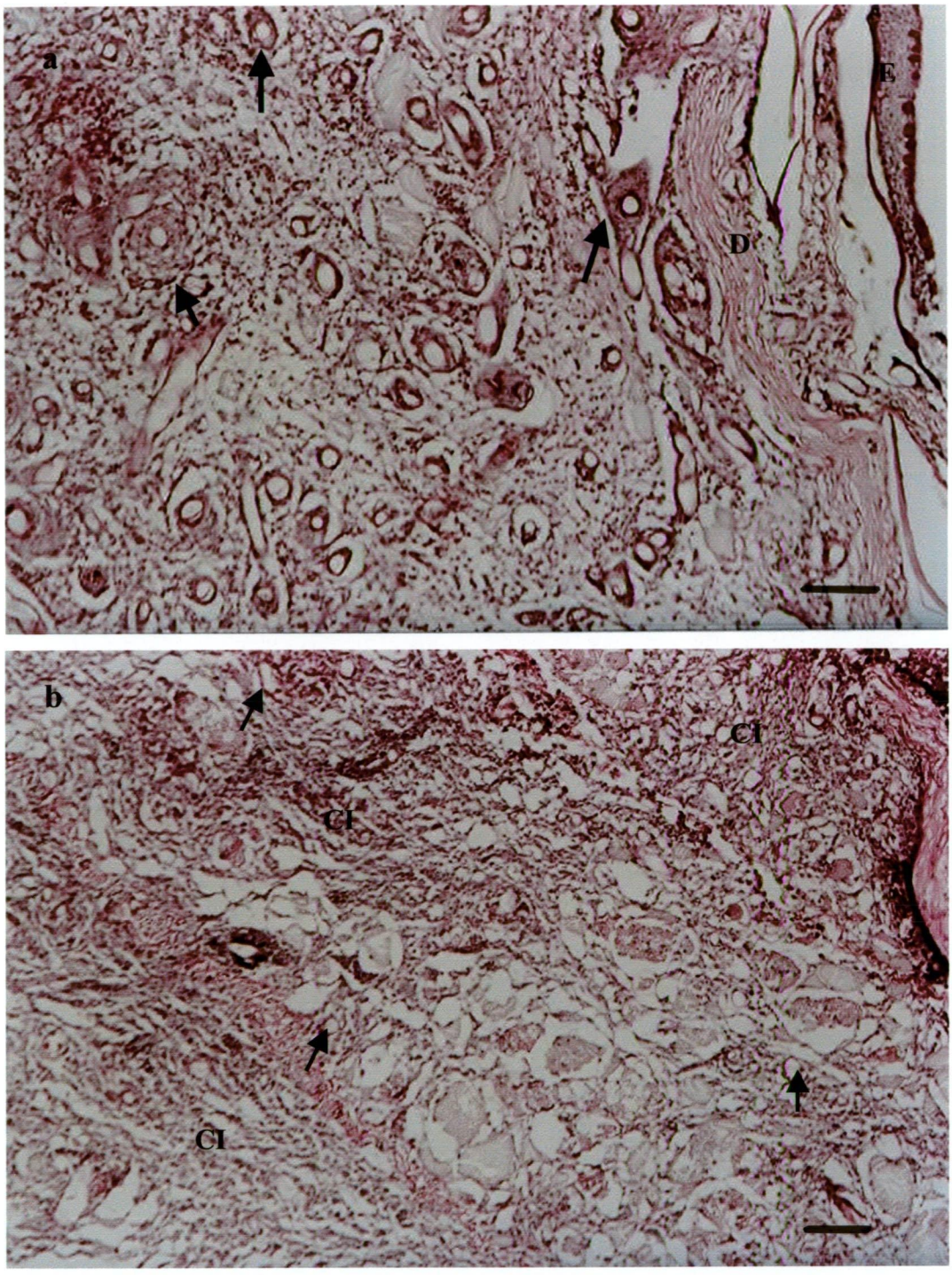


Figure 5.4 Photomicrographs of sand whiting at day 10 p.i. **a) high temp**: epidermis (E) inflamed, necrotising dermatitis (D) with mycotic granulomas, remarkable granulomatous response (arrows) in the muscle area; **b)gradual temp drop**: some fungal hyphae (arrows) present but no significant granuloma formation, marked inflammatory cell response (CI). (PAS stain, bar=100 μm)

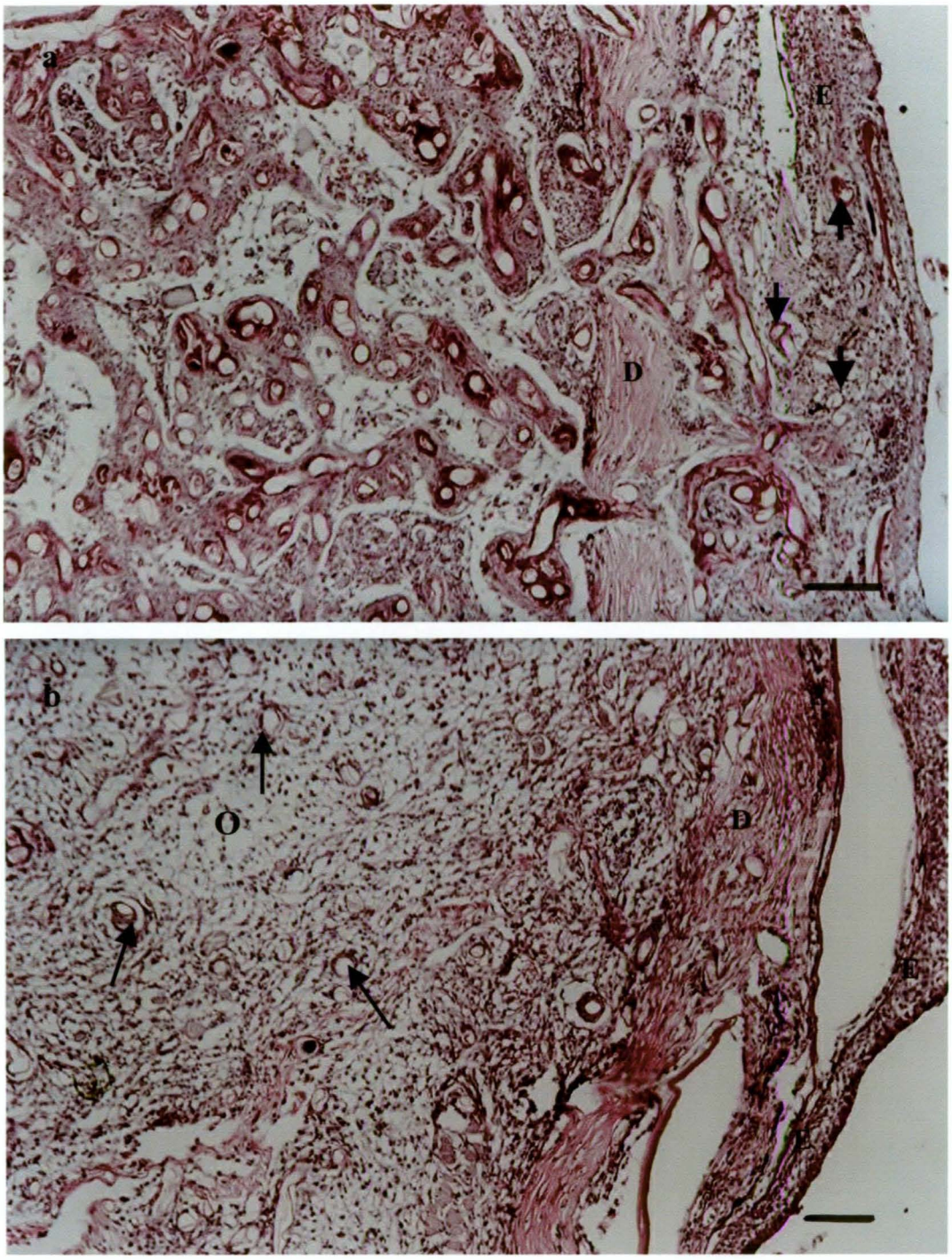


Figure 5.5 Photomicrographs of sand whiting at day 14 p.i. **a) high temp**: epidermis (E) highly inflamed with fungal invasion (arrows), dermis (D) and muscle area with linked granulomas due to fibrosis. **b)gradual temp drop**: epidermis(E) and dermis (D) inflamed with fungal invasion (arrows) but limited granuloma formation, marked myonecrosis and oedema (O). (PAS stain, bar=100 μm)

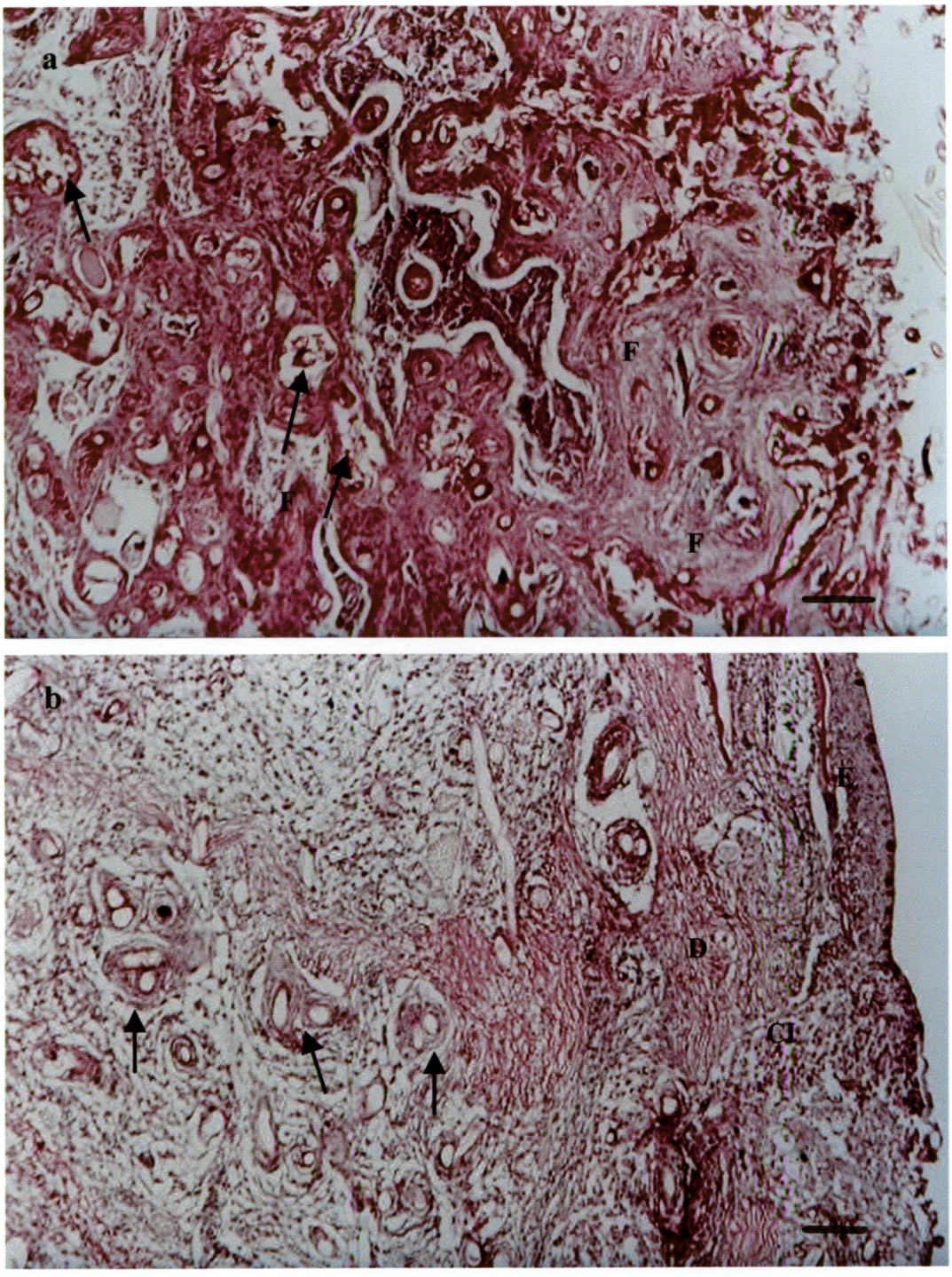


Figure 5.6 Photomicrographs of sand whiting at day 16 p.i. **a) high temp**: epidermis and dermis eroded, extensive fibrosis (F), fibrous granulomas with fungal debris (arrows) in the centre; **b) rapid temp drop**: epidermis(E) and dermis (D) highly inflamed (CI) and necrotic, thicker granulomas formed (arrows). (PAS stain, bar=100 μm)

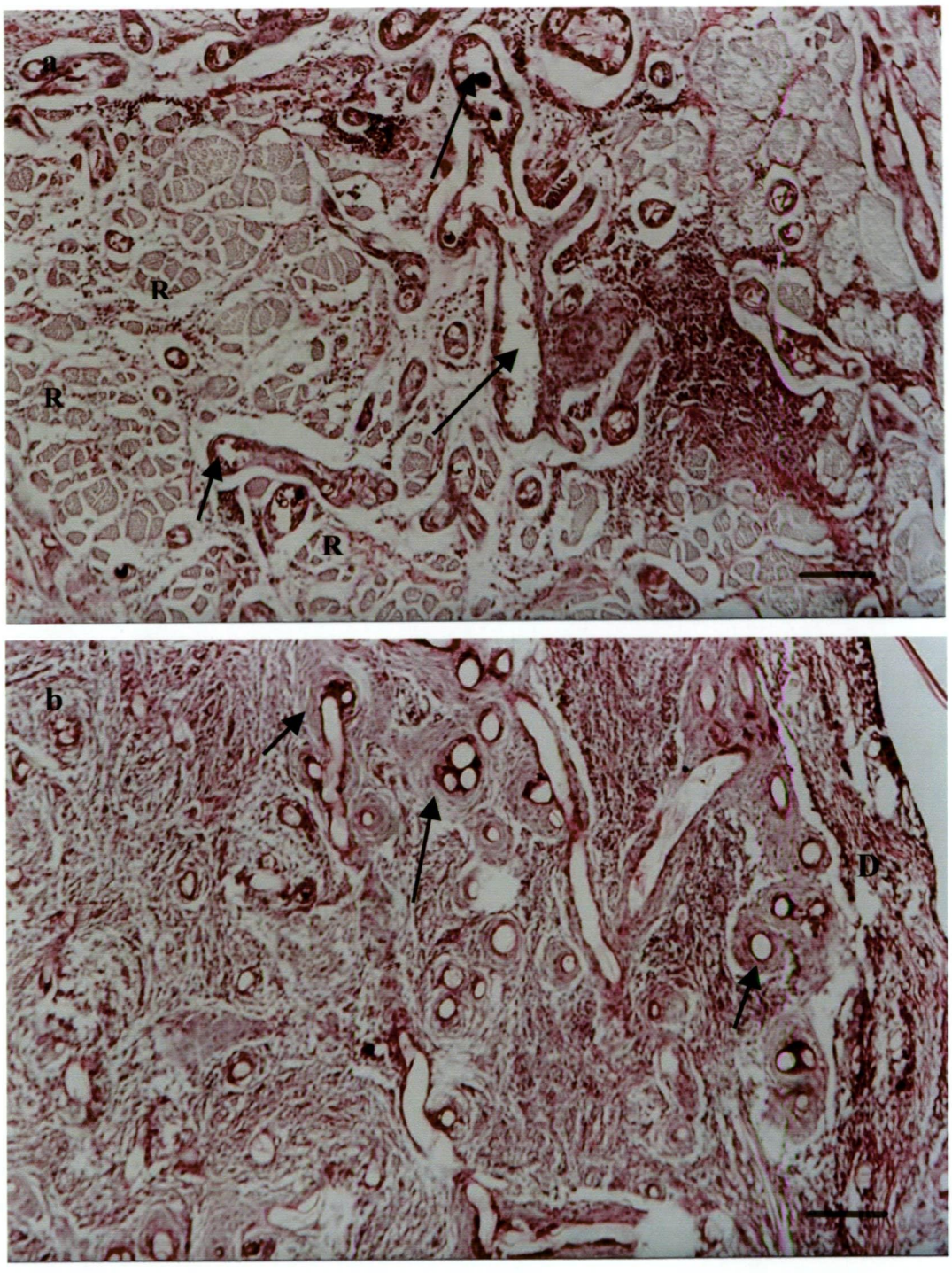


Figure 5.7 Photomicrographs of sand whiting at day 18 p.i. **a) high temp**: resolution of lesion with regeneration (R) of muscle fibres and fungal hyphae completely lysed (arrows) within granulomas; **b) gradual temp drop**: necrotising dermatitis (D), periphery of fungal granulomas fibrotic (arrows) with some granulomas linked together. (PAS stain, bar=100 μm)

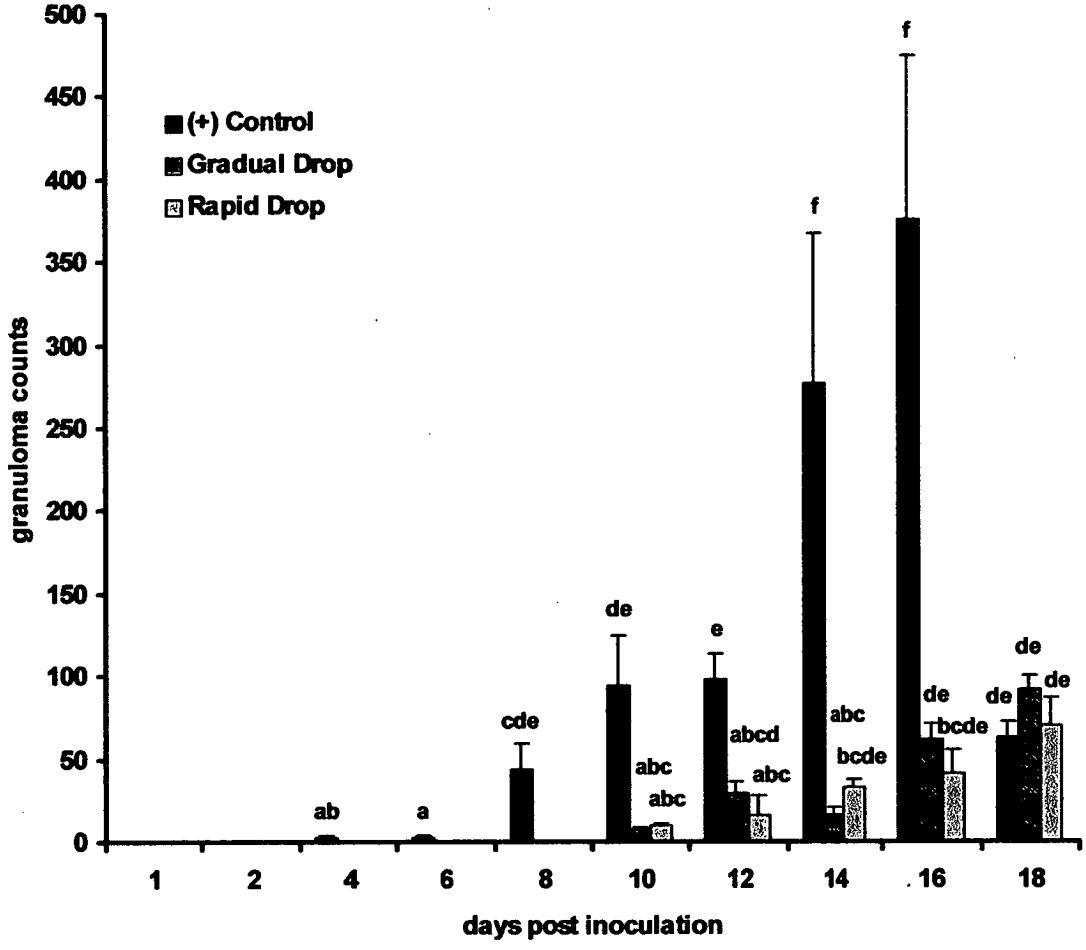


Figure 5.8 Mycotic granuloma counts in *A. invadans*-infected sand whiting fed with high fish oil diet and subjected to temperature variations. Bars are means ± s.e. of three replicates; common letters not significantly different by Tukey-Kramer HSD test at $P < 0.05$.

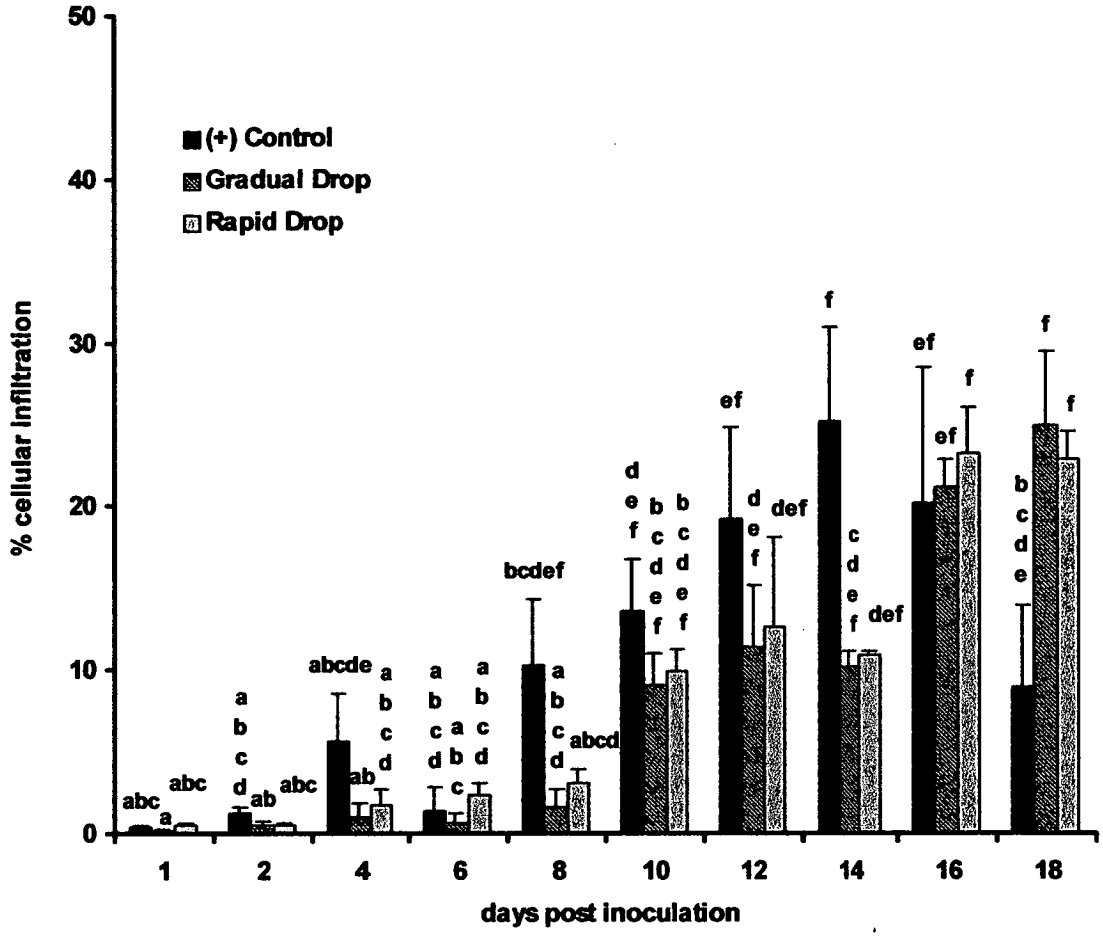


Figure 5.9 Percentage cellular infiltration in *A. invadans*-infected sand whiting fed with a high fish oil diet and subjected to temperature variations. Bars are means ± s.e. of three replicates; common letters not significantly different by Tukey-Kramer HSD test at $P < 0.05$.

There was a significantly higher cellular infiltration at day 8 p.i. in fish held at 26 °C than in fish held at 17 °C. At days 4, 8,,10, 12 and 14 p.i., fish held at 26 °C had higher percentage of cellular infiltration, although not significantly, than the fish held at 17 °C. At day 18 p.i., fish kept at lower temperature had significantly higher percentage of cellular infiltration than the fish kept at 26 °C.

Effects of fish oil supplementation

Three factor ANOVA of the granuloma counts showed significant differences between treatments due to sampling day (P=<0.001), temperature (P=<0.001), level of fish oil (P=0.0023) and the interaction of sampling day and temperature. Table 5.5 shows the mean granuloma counts for each experimental treatment at each sampling day. There was no significant difference in the granuloma counts taken from either the rapid or gradual temperature drop treatments (both low and high fish oil diets) which were consistently lower than the counts from the high temperature treatment, except at day 18 p.i. Fish fed with a low fish oil diet, especially the (+) fish, generally had higher granuloma counts than the fish fed with a high fish oil diet. However, the difference between the two (+) control groups was significant only at day 6 p.i.

Significant differences were also detected between treatments in terms of the percentage of cellular infiltration. These were due to the level of fish oil (P=<0.001), sampling day (P=<0.001), temperature (P=<0.001) and the interaction of sampling day and temperature (P=<0.001). Table 5.6 shows the mean % cellular infiltration for each treatment at each sampling day. The data from the (-) control fish were not included in the analysis since cellular infiltration response was very minimal. No significant difference was detected in the cellular infiltration percentage of fish fed with low or high fish oil diet and subjected to either rapid or gradual temperature drop. At days 6 and 14 p.i., (+) control fish fed with low fish oil diet had significantly higher percentage of cellular infiltration than the (+) control fish fed with high fish oil diet.

Table 5.5 Mycotic granuloma counts in *A. invadans*-injected sand whiting. Values are means ± s.e. of three replicates; means followed by a common letter within a row (sampling day) are not significantly different by Tukey-Kramer HSD test at $P < 0.05$.

Sampling day (p.i.)	Low fish oil diet			High fish oil diet		
	(+) Control	Gradual temp drop	Rapid temp drop	(+) Control	Gradual temp drop	Rapid temp drop
4	5.0±2.5^a	0^a	0^a	3.0±1.0^a	0^a	0^a
6	13.7±4.4^a	0^b	0^b	2.0±2.0^b	0^b	0^b
8	65.3±18.3^a	2.0±0^b	2.0±2.0^b	43.0±16.1^a	0^b	0^b
10	90.0±21.7^a	15.7±4.9^b	11.7±8.0^b	93.7±30.7^a	7.0±1.5^b	10.0±0.6^b
12	191.3±40.3^a	11.3±6.6^b	20.7±6.4^b	97.3±15.8^a	29.3±7.4^b	15.3±12.0^b
14	353.3±56.2^a	26.7±7.7^b	39.3±9.0^b	276.7±89.9^a	15.7±4.8^b	32.7±4.7^b
16	456.0±64.6^a	70.3±17.7^b	84.7±22.3^b	376.0±99.0^a	61.0±9.9^b	41.3±14.4^b
18	75.0±21.2^a	88.7±10.7^a	94.7±5.8^a	62.3± 9.8^a	91.3±9.0^a	69.5±17.5^a

Table 5.6 Percentage cellular infiltration in *A. invadans*-injected sand whiting. Values are means ± s.e. of three replicates; means followed by a common letter within a row (sampling day) are not significantly different by Tukey-Kramer HSD test at $P < 0.05$.

Sampling days (p.i.)	Low fish oil diet			High fish oil diet		
	(+) Control	Gradual temp drop	Rapid temp drop	(+) Control	Gradual temp drop	Rapid temp drop
1	0.5±0.12^{a}	0.1±0.01^{a}	0.3±0.12^{a}	0.4±0.03^{a}	0.13±0.13^{a}	0.5±0.09^{a}
2	2.6±0.5^{a}	1.0±0.5^{a}	1.0±0.3^{a}	1.3±0.30^{a}	0.5±0.23^{a}	0.5±0.13^{a}
4	10.0±5.4^{a}	1.4±0.1^{a}	0.7±0.3^{a}	5.6±3.0^{a}	0.9±0.9^{a}	1.7±1.0^{a}
6	7.8±0.4^{a}	4.6±2.2^{ab}	1.4±0.14^{ab}	1.4±1.4^{b}	0.7±0.6^{b}	2.4±0.7^{ab}
8	24.2±5.7^{a}	6.7±2.6^{b}	4.0±1.4^{b}	10.3±4.0^{ab}	1.6±1.0^{b}	3.0±0.9^{b}
10	18.5±3.5^{a}	8.2±4.2^{a}	7.4±1.8^{a}	13.6±3.1^{a}	9.0±2.0^{a}	9.9±1.4^{a}
12	25.6±3.9^{a}	10.3±6.3^{a}	15.6±2.8^{a}	19.2±5.6^{a}	11.4±3.8^{a}	12.6±5.5^{a}
14	44.4±5.5^{a}	18.2±1.9^{bc}	19.7±2.5^{bc}	25.2±5.7^{b}	10.2±0.90^{c}	10.9±0.17^{bc}
16	30.7±2.8^{a}	16.7±1.7^{a}	24.2±5.4^{a}	20.2±8.4^{a}	21.2±1.7^{a}	23.2±2.8^{a}
18	16.6±6.4^{ab}	35.1± 4.2^{a}	29.1±1.8^{a}	8.9±5.0^{b}	25.0±4.6^{ab}	22.8±1.7^{ab}

5.3.2 Effects of L-cysteine ethyl ester

5.3.2.1 Fish mortality

Table 5.7 shows the fish mortality in sand whiting treated with L-CEE and in other experimental treatments simultaneously performed and presented in Chapter Three. There was very limited mortality during the week-long acclimation period and during the first week of the trial except for all the fish in one tank (one replicate) which died when the temperature was decreased gradually from 26 °C to 17 °C. Consequently, there was only one replicate for this treatment during the 12-day experimental period.

5.3.2.2 Gross observations and histopathology

As mentioned in Chapter Three (section 3.3.2.3), most of the moribund and dead fish during the experiment exhibited petechial hemorrhages on the dorsum immediately below the dorsal fin, and these signs were observed in some L-CEE-treated fish as well.

Histopathology examination showed inflammatory cell infiltration, which was likely associated with epidermal erosion. One moribund fish held at 26 °C (tank 4) during the main experimental period had fungal hyphae and bacteria associated with skin hemorrhages. However, skin hemorrhages from other moribund and dead fish from the replicate tank (tank 7) and the tank kept at 19 °C were free from any bacterial or fungal contamination. Some fish also exhibited spongy and vacuolated epidermis as in the sampled fish from other experimental treatments

Statistical analysis of the epidermal thickness measurements and mucous cell counts did not show any significant difference between the treatments [(+) control, cohabitation and L-CEE] used in the experiment. Table 5.8 shows the quantitative data obtained from the experiment. There was low mucous cell counts, especially in samples which exhibited epidermal spongiosis and oedema.

Table 5.7 Fish mortality in L-CEE-supplemented sand whiting, including the mortality in three other experimental treatments presented in Chapter Three.

Tank no.	Mortality during acclimation (one week)	Temperature treatments	Mortality during week 1 of trial	Experimental treatments	Mortality during main experiment (12 days)
1	0/12	26 °C	2/12	Fungal mats only	0/10
2	2/12	26 °C	5/10	Cohabitation + mats	0/5
3	1/12	26 °C	1/11	Fungal mats only	2/10
4	0/12	26 °C	2/12	L-CEE + mats	1/10
5	1/12	26 °C	1/11	Abrasion + mats	2/10
6	1/12	26 °C	9/11	Cohabitation + mats	2/2
7	4/12	26 °C	1/8	L-CEE + mats	2/7
8	2/12	26 °C	2/10	Abrasion + mats	2/8
9	1/12	26-19 °C	0/11	L-CEE + mats	4/11
10	1/12	26-19°C	1/11	Cohabitation + mats	5/10
11	1/12	26-19 °C	2/11	Abrasion + mats	7/9
12	2/12	26-19 °C	3/10	Cohabitation + mats	5/7
13	5/12	26-19 °C	2/7	Fungal mats only	1/5
14	2/12	26-19 °C	2/10	Fungal mats only	4/8
15	1/12	26-19 °C	11/11	L-CEE + mats	-
16	0/12	26-19 °C	0/12	Abrasion + mats	9/12

Table 5.8 Epidermal thickness measurements and mucous cell counts in sand whiting taken from one scale unit per sample of fish. Values for the two different treatments [cohabitation and (+) control] were included as reference for the L-CEE data. Values are means ± s.e. of 2 replicates. (* Values based on one replicate only due to fish mortality).

	Sampling day	Treatments		
		(+) Control	Cohabitation	L-CEE
Epidermal thickness (μm)				
26 °C	**1**	61.2 ± 10.6	63.8*	57.2 ± 5.3
	3	58.6 ± 5.2	33.8*	52.3 ± 23.9
	6	66.4 ± 6.4	72.8*	49.7 ± 5.9
	9	49.2 ± 1.1	50.3*	44.2 ± 0.4
19 °C	**1**	46.0 ± 4.1	55.9 ± 6.2	64.7*
	3	39.2 ± 23.6	86.7 ± 18.9	135.8*
	6	100.6 ± 20.3	86.9*	75.0*
	9	44.0 ± 8.1	144.1*	33.4*
Mucous cell counts				
26 °C	**1**	2.5 ± 0.5	1*	5.5 ± 5.5
	3	0	0*	1.5 ± 1.5
	6	0	0*	2.0 ± 1.0
	9	0.5 ± 0.5	2*	2.5 ± 0.5
19 °C	**1**	2.5 ± 0.5	2.0 1.0	3.0*
	3	0	0.5 0.5	4.0*
	6	3.0 ± 3.0	0*	3.0*
	9	3.0 ± 3.0	2.0*	0*

5.3.3. Effects of levamisole and yeast glucan

5.3.3.1 Water quality

Table 5.9 shows the basic water quality variables that were monitored during the 8-day experimental period of the four immunomanipulation studies which involved oral administration of levamisole and a yeast glucan preparation. NH_3-N level was <0.1 mgL^{-1} in Experiment I.A, <0.25 mgL-1 in Experiment I.B and was not detectable with the measuring kit in Experiments II.A and II.B.

Table 5.9 Water quality in the treatment tanks used in the immunostimulation experiments. Values are means ± s.e of two replicates per treatment.

Treatments	Salinity (‰)	Temperature (°C)	Dissolved oxygen (mgL^{-1})
Experiment I.A			
(-) control	5.3 ± 0.4	26.2 ± 0.1	7.3 ± 0.4
(+) control	6.0 ± 0.9	26.2 ± 0.4	6.3 ± 0.2
Levamisole	5.8 ± 0.6	25.8 ± 0.2	7.0 ± 0.2
Yeast glucan	5.7 ± 0.6	25.7 ± 0.2	6.0 ± 0.4
Experiment I.B			
(-) control	5.8 ± 0.4	17.2 ± 0.2	8.6 ± 0.7
(+) control	5.0 ± 0	17.1 ± 0.3	8.3 ± 0.4
Levamisole	5.0 ± 0	17.3 ± 0.2	8.7 ± 0.2
Yeast glucan	5.0 ± 0	17.2 ± 0.3	8.8 ± 0.4
Experiment II.A			
(-) control	5.0 ± 0	26.0 ± 0.2	3.8 ± 0.1
(+) control	5.0 ± 0	25.8 ± 0.2	4.2 ± 0.3
Yeast glucan	5.0 ± 0	25.8 ± 0.6	4.3 ± 0.2
Experiment II.B			
(-) control	8.2 ± 0.4	19.2 ± 0.1	6.9 ± 0.6
(+) control	8.0 ± 1.0	19.2 ± 0.2	7.0 ± 0.7
Yeast glucan	7.8 ± 0.4	19.0 ± 0.1	5.7 ± 0.7

Experiment I.A: Immunostimulation with levamisole and yeast glucan at 26 °C.

Experiment I.B: Immunostimulation with levamisole and yeast glucan with gradual temp decrease.

Experiment II.A: Immunostimulation with yeast glucan at 26 °C.

Experiment II.B: Immunostimulation with yeast glucan with gradual temp decrease.

5.3.3.2 Gross signs and fish mortality

The inoculation sites were usually pale-coloured 24-hr p.i. For the PBS- or APW-injected fish or (-) control, the discolouration disappeared after 3-4 days p.i. But for the zoospore-injected fish, the discoloured sites had pinpoint hemorrhages and were slightly inflamed (Figure 5.10) at day 5 p.i. From day 6-8 p.i., the inoculation sites were highly inflamed and the fish swim lethargically at the surface of the water.

Mortality only occurred in the (-) control group (1 fish in replicate 1 and 3 fish in replicate 2) of sand whiting kept at 26 °C since they were able to slip through between the screen covers of the tanks and jumped out. No mortality occurred in fish subjected to gradual temperature drop and maintained at 17 °C. In the second batch of trials, mortality occurred in (+) control fish (2 fish in replicate 1 and 3 fish in replicate 2) during the experiment while 1 fish from the (-) control fish died 24 hours after PBS inoculation. In fish subjected to gradual temperature drop and maintained at 19 °C, 3 fish from the (+) control group died and only 1 fish died from the glucan-treated group.

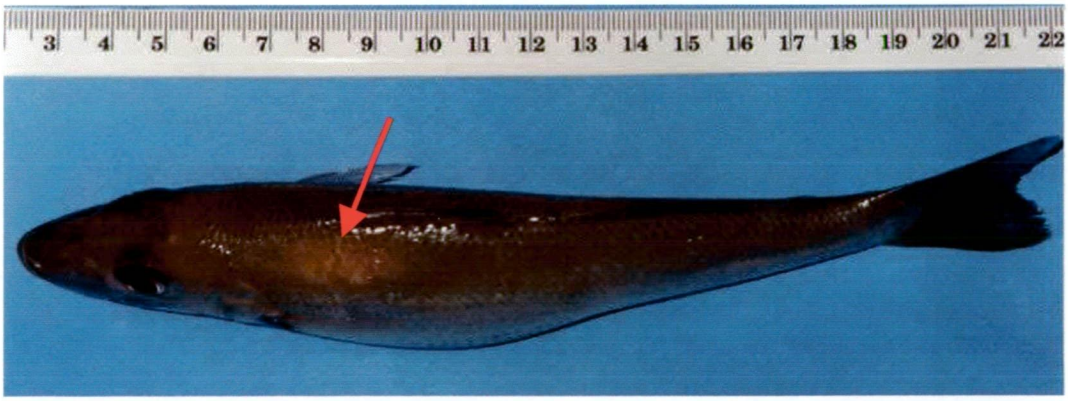


Figure 5.10 Sand whiting inoculated with zoospores showed pale-coloured, slightly-inflamed injection site (arrow) with petechial hemorrhages.

5.3.3.3 Haematological indices

Significant difference was detected in haematocrit levels due to temperature and treatments. Haematocrit was higher in fish held at 17 °C than in fish maintained at 26 °C. Figures 5.11 (a & b) show that immunostimulated fish at 26 °C had significantly higher haematocrit than the (+) control using one-way ANOVA and mean comparison at each temperature levels. However, there was no significant difference between the treatments in fish held at 17 °C.

Polymorphonuclear neutrophils (PMN) and thrombocytes were not significantly different between treatments. However, significant difference was detected in lymphocytes due to temperature as fish held at 17 °C were higher than in fish held at 26 °C. In fish held at 26 °C, (-) control fish had significantly higher lymphocytes than the (+) control fish.

Significant difference in monocytes was due both to temperature and treatments and their interaction. Fish held at 17 °C had lower monocytes than the fish held at 26 °C. In fish held at 26°C, the (+) control fish had significantly higher monocytes than the (-) control but levamisole- and glucan-treated fish did not show any significant difference with the (+) control fish. Glucan- and levamisole-treated fish had higher monocytes than the (+) control in fish kept at 17 °C. Table 5.10 shows the haematological indices compared in the two trials. Figure 5.12 (a & b) shows the comparison of the treatments at each temperature level.

5.3.3.4 Nonspecific immune parameters

Table 5.11 shows the non-specific immune parameters measured in sand whiting infected with *A. invadans* and kept either at 26 °C or 17 °C. Glucan- and levamisole-treated fish had higher phagocytic activity but were not significantly different from the (+) control. (Figures 5.13 a & b).

Table 5.10 Haematological indices. Values are means ± s.e. of two replicates, common letters within a column not significantly different by Tukey-Kramer HSD test at P=<0.05.

Treatments	Haematocrit (%)	Lymphocytes (%)	Monocytes (%)	PMN (%)	Thrombo-cytes (%)
26 °C					
(-) control	26.0 ± 0.3^{ab}	46.7 ± 7.2^{bc}	7.2 ± 0.6^{a}	4.2 ± 1.7^{a}	41.8 ± 8.4^{a}
(+) control	23.4 ± 0.6^{a}	23.6 ± 3.6^{a}	44.6 ± 1.0^{c}	7.6 ± 1.2^{a}	24.2 ± 3.5^{a}
Levamisole	34.3 ± 3.0^{abc}	30.7 ± 0.05^{ab}	28.8 ± 7.4^{c}	12.8 ± 3.2^{a}	28.0 ± 10.4^{a}
Glucan	35.8 ± 2.3^{bc}	39.6 ± 2.6^{abc}	24.4 ± 0.4^{bc}	9.2 ± 2.2^{a}	26.6 ± 1.0^{a}
Gradual temp drop					
(-) control	38.0 ± 1.6^{bc}	55.7 ± 0.5^{c}	4.5 ± 1.3^{a}	4.8 ± 2.8^{a}	35.1 ± 4.7^{a}
(+) control	45.3 ± 2.5^{c}	56.0 ± 4.0^{c}	7.2 ± 1.4^{a}	11.6 ± 3.0^{a}	24.0 ± 1.0^{a}
Levamisole	44.1 ± 1.9^{c}	54.0 ± 1.4^{c}	12.8 ± 0.5^{ab}	10.0 ± 2.2^{a}	22.4 ± 0.2^{a}
Glucan	40.2 ± 3.5^{c}	50.4 ± 5.0^{bc}	12.1 ± 1.4^{ab}	14.5 ± 2.7^{a}	20.0 ± 0.8^{a}

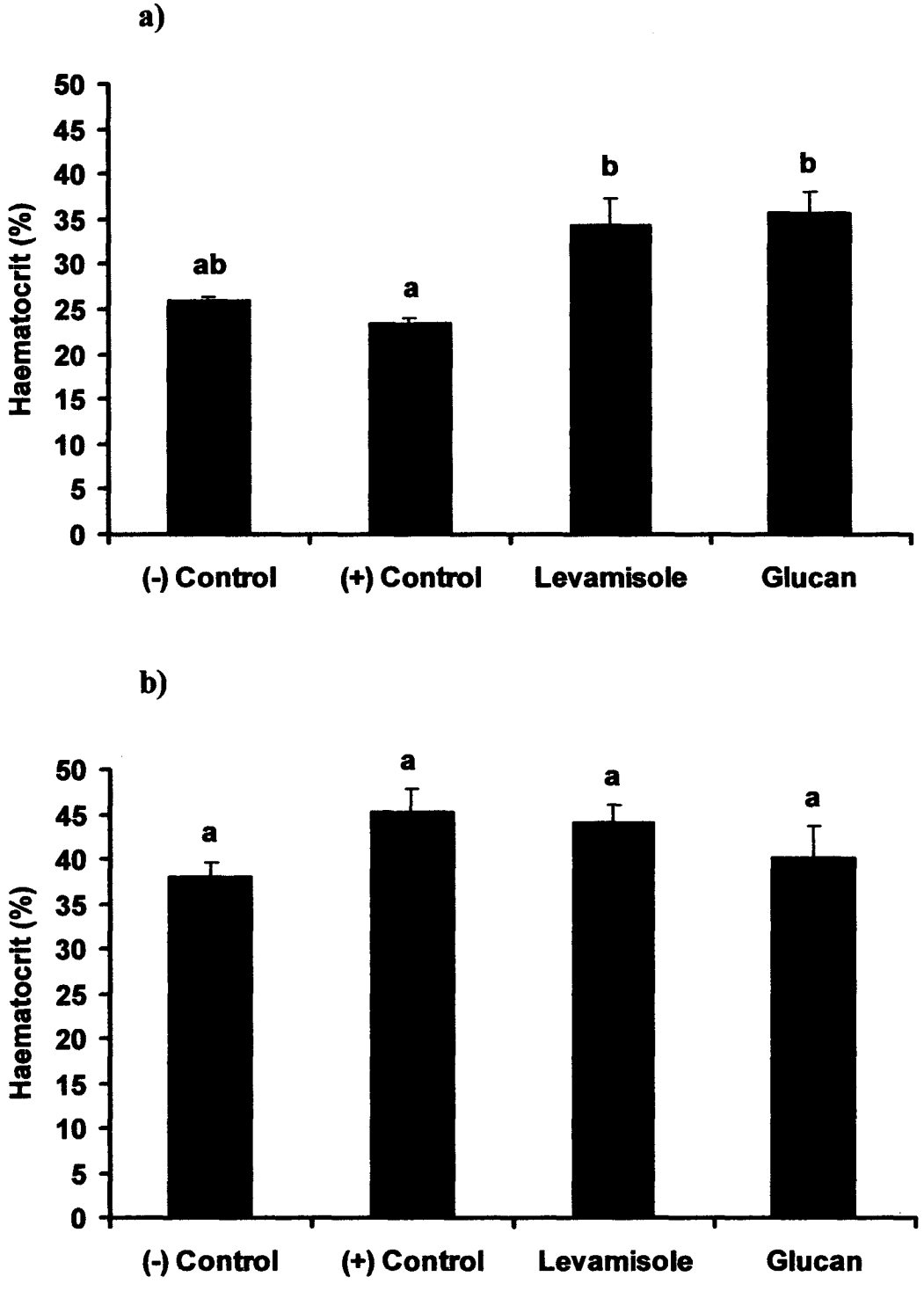


Figure 5.11 Haematocrit levels in sand whiting held at **a) 26 °C** and **b) 17 °C**. Bars are means ± s.e.; common letters not significantly different by Tukey-Kramer HSD test at P= <0.05.

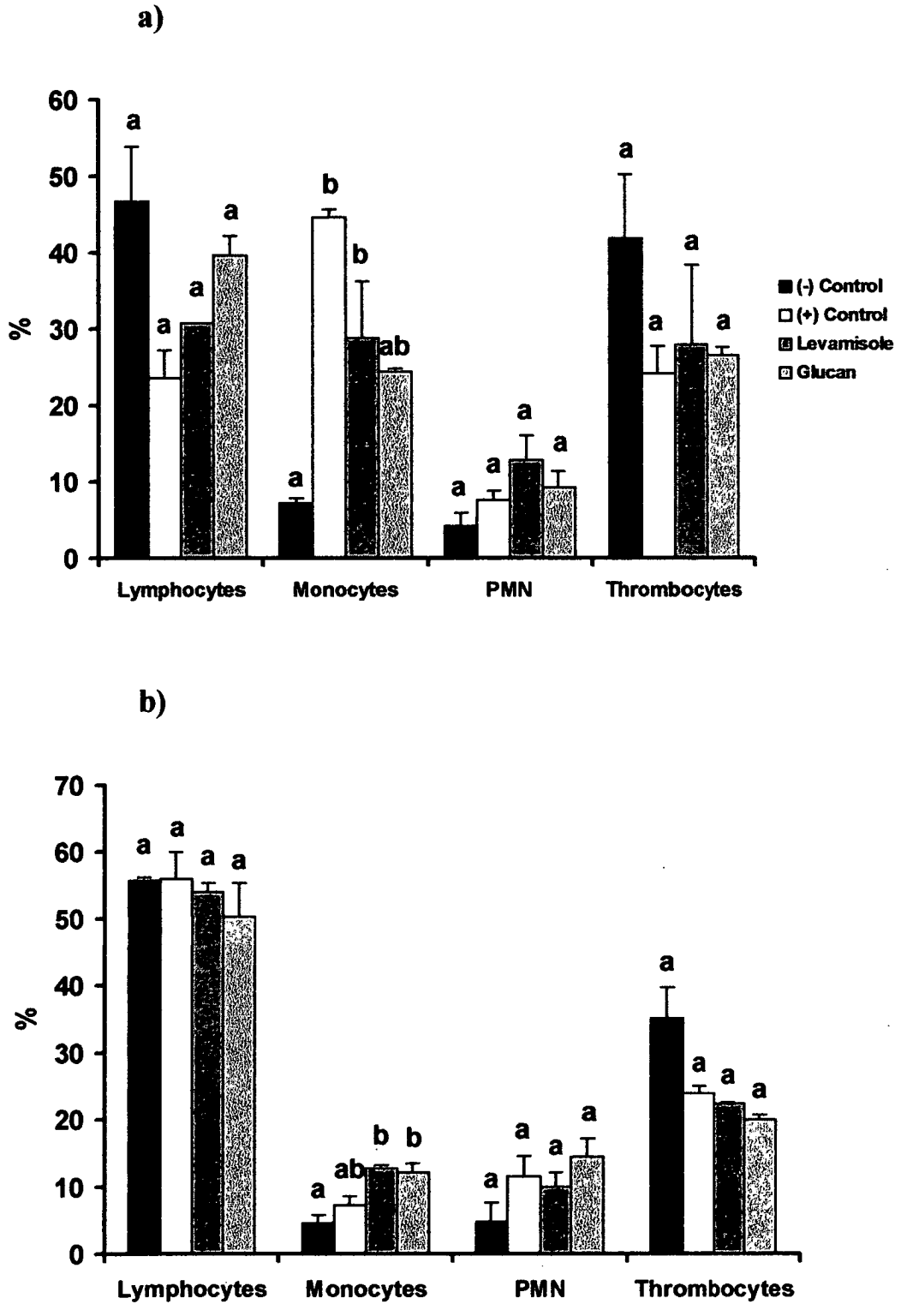


Figure 5.12 Differential WBC counts in sand whiting held at **a)26°C** and **b)17 °C**. Bars are means ± s.e; common letters within same cell type or group not significantly different by Tukey-Kramer HSD test at P=< 0.05

No significant difference was detected in the lysozyme levels but levamisole-treated fish showed higher activity than the (+) control at 26 °C and at 17 °C. Significant difference in the antiprotease activity was due to temperature, treatments and their interaction. The antiprotease level in the (-) control fish at 17 °C was significantly lower than in the (-) control fish held at 26 °C. Levamisole-treated fish showed significantly higher antiprotease level than the (+) control at 26 °C. One-way ANOVA and comparison of means from the low temperature trial showed that levamisole-treated fish had significantly higher antiprotease level than the (+) control. (Figures 5.15 a & b).

In Experiments II.A and II.B, significantly higher extracellular production of O_2^- detected in fish held at 26 °C than in fish held at l9 °C. At 60 min and 120 min timepoints, the (-) control group showed significantly higher extracellular O_2^- production than the (+) control fish. Glucan treated fish showed significantly higher extracellular O_2^- production than the (+) control fish. (Figures 5.16 a & b). The (-) control fish held at 19 °C had significantly higher alternative complement (ACP) activity than the (-) fish at 26 °C. The (+) control fish had significantly higher ACP activity than the (-) control and glucan-treated fish at 26 °C but at low temperature, the (+) control fish did not show any significant difference in comparison with the (-) control and the glucan-treated fish. (Figure 5.17)

5.3.3.5 Histopathology

Very minimal inflammatory response was seen in PBS- and APW-injected fish. Zoospore-injected fish showed typical mycotic granulomas and intense inflammatory cell infiltration in the muscle tissue. Statistical analysis of the differential granuloma counts detected differences between treatments in the Stage I (active) and Stage III (lysed) granulomas due to temperature and the treatment. Fish infected with *A. invadans* and held at 19 °C had higher Stage I granulomas than the other 3 treatments at day 8 p.i. Stage III granulomas were significantly higher in glucan-treated fish at 26 °C and at 19 °C. No significant difference was detected between treatments in terms of the percentage of Stage II granulomas. (Figure 5.18)

Table 5.11 Nonspecific immune parameters. Values are means ± s.e. of two replicates, common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

Treatments	Phagocytic activity (%)	Lysozyme (µg/ml)	Antiprotease activity (no. of trypsin units inhibited/µL plasma)
26 °C			
(-) control	41.2±1.2^{a}	11.0±4.1^{a}	41.4±1.9^{b}
(+) control	44.7±3.2^{ab}	8.2±1.4^{a}	45.9±0.6^{bc}
Levamisole	48.2±3.2^{ab}	15.6±4.4^{a}	54.5±0.6^{de}
Glucan	56.8±0.8^{ab}	7.0±1.9^{a}	49.3±0.8^{cd}
Gradual temp drop (26°C –19°C)			
(-) control	41.0±1.4^{a}	6.1±0.3^{a}	4.7±0^{a}
(+) control	44.0±1.6^{ab}	9.2±1.4^{a}	57.6±0.8^{ef}
Levamisole	59.8±2.0^{b}	11.6±1.2^{a}	62.8±1.2^{f}
Glucan	53.6±6.2^{ab}	9.9±1.6^{a}	59.4±1.0^{ef}

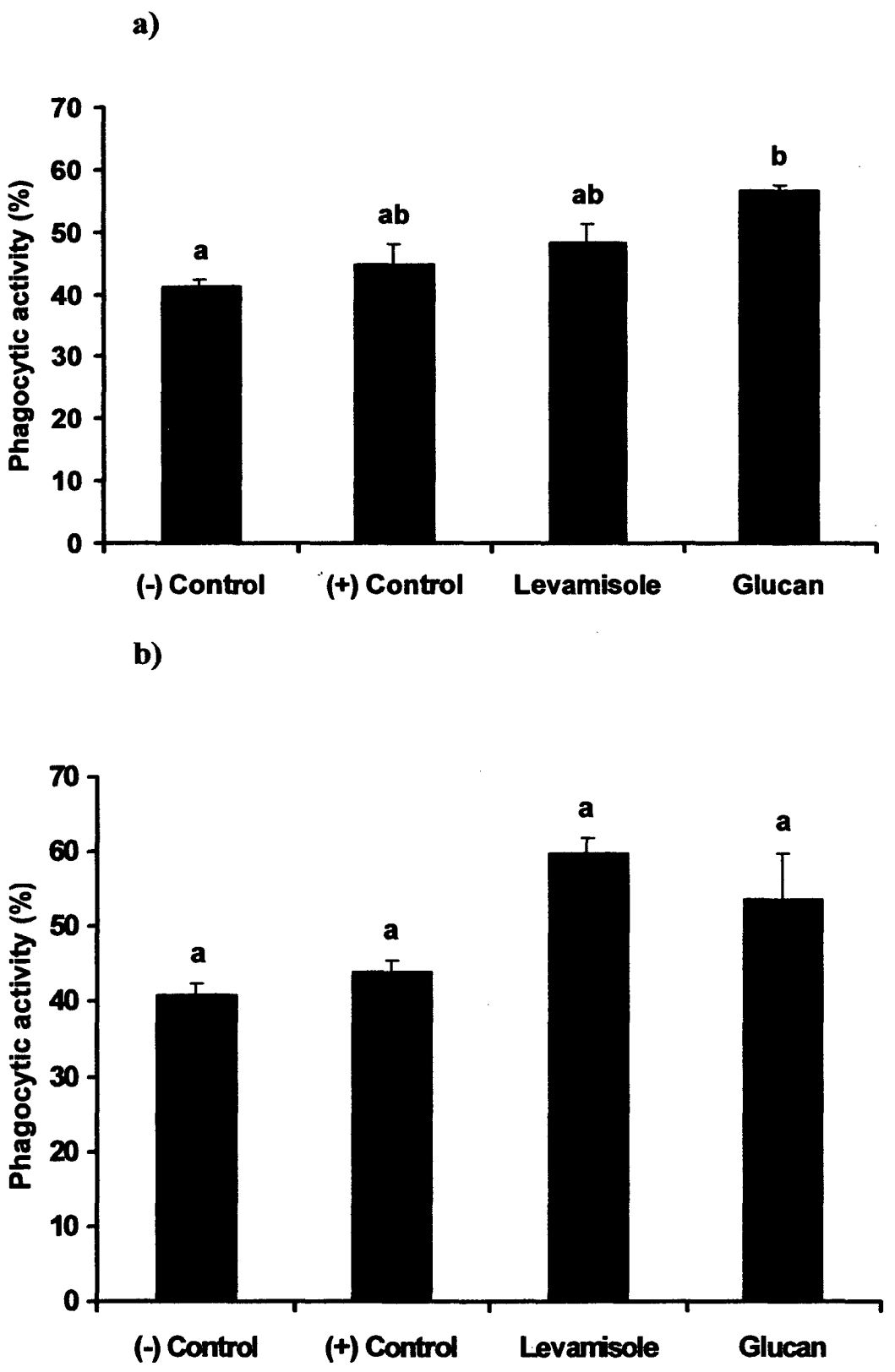


Figure 5.13 Phagocytic activity in sand whiting held at **a) 26 °C** and **b) 17 °C**. Bars are means ± s.e.; common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

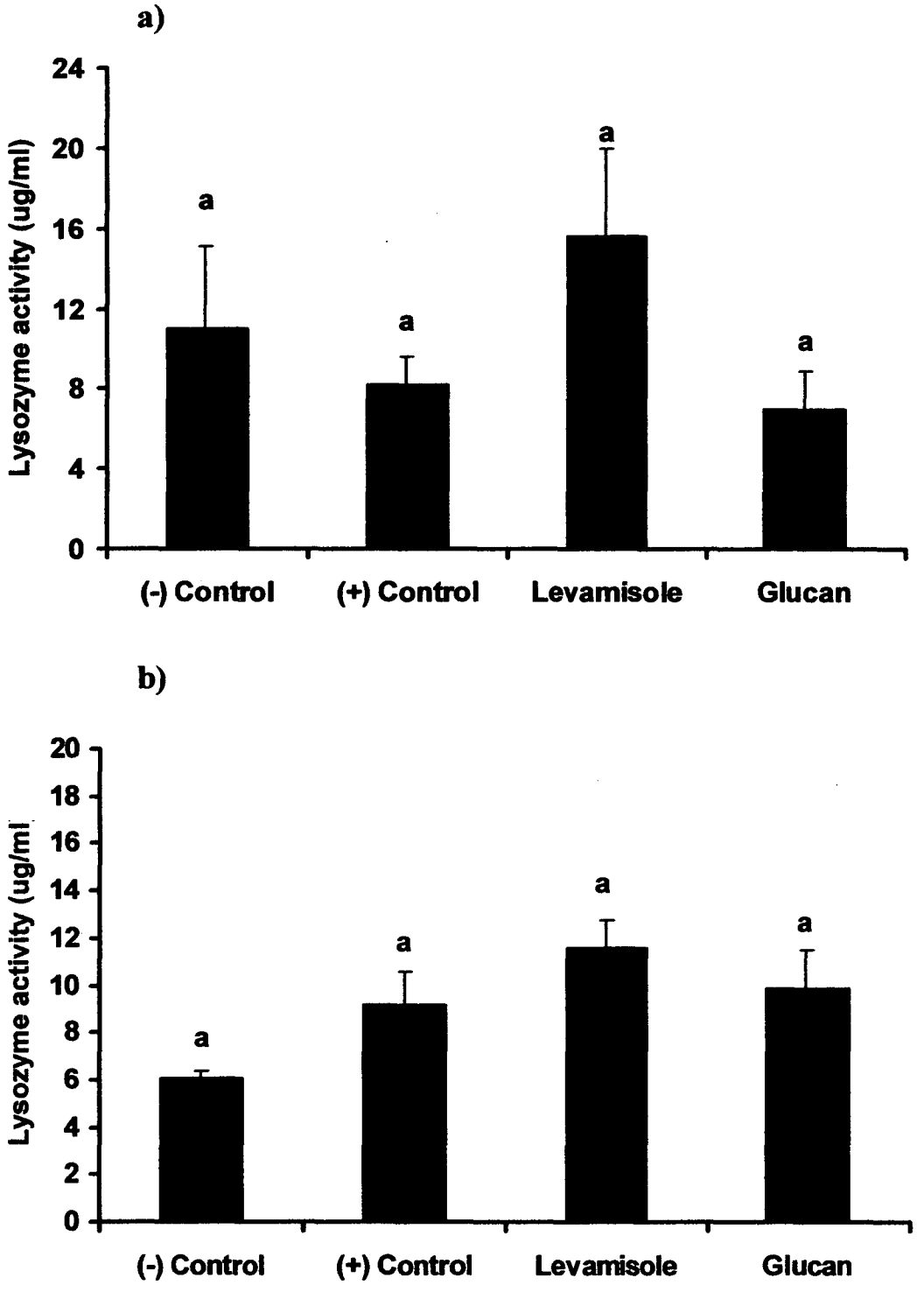


Figure 5.14 Lysozyme activity in sand whiting held at **a)** 26 °C and **b)** 17 °C. Bars are means ± s.e.; common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

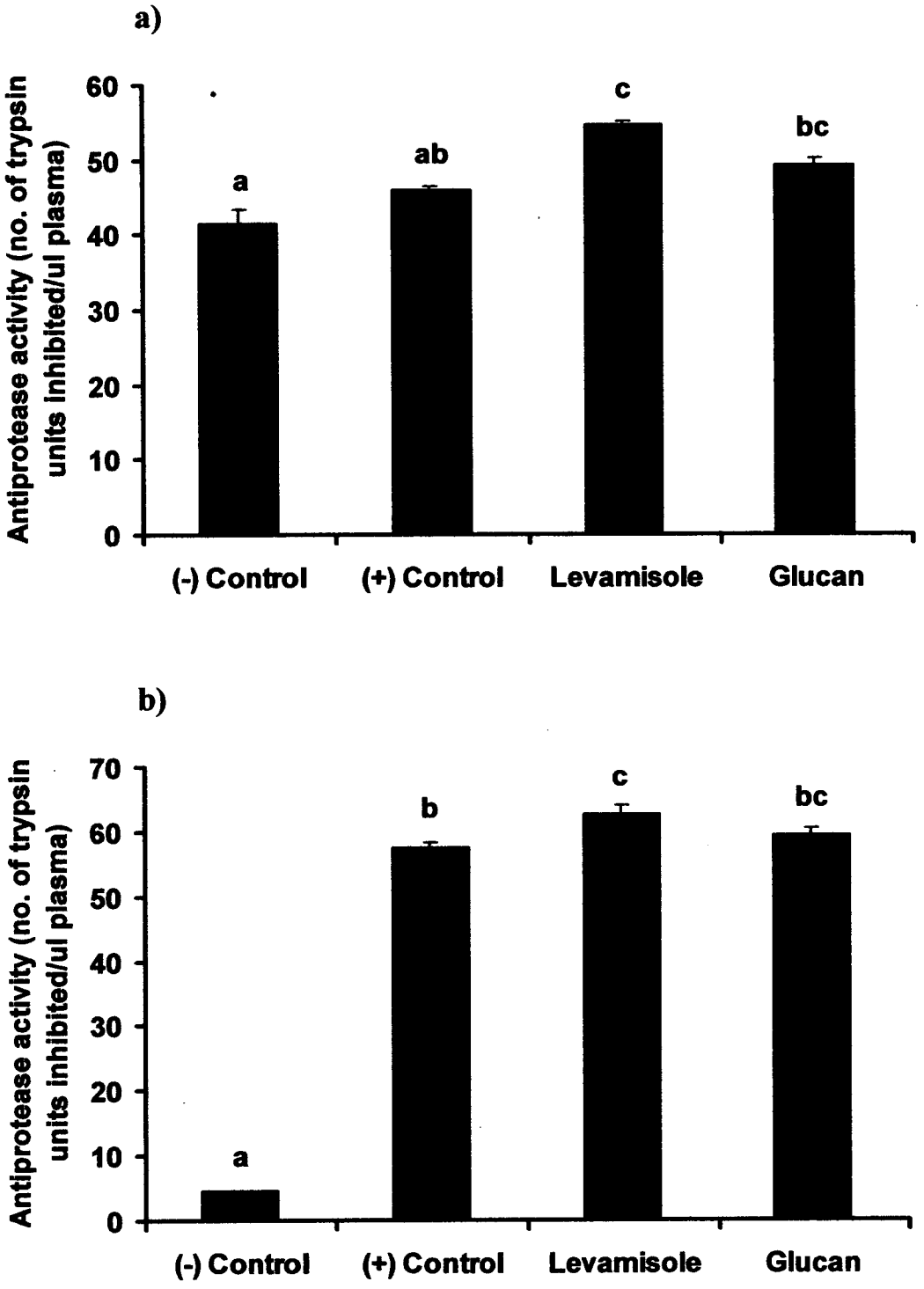
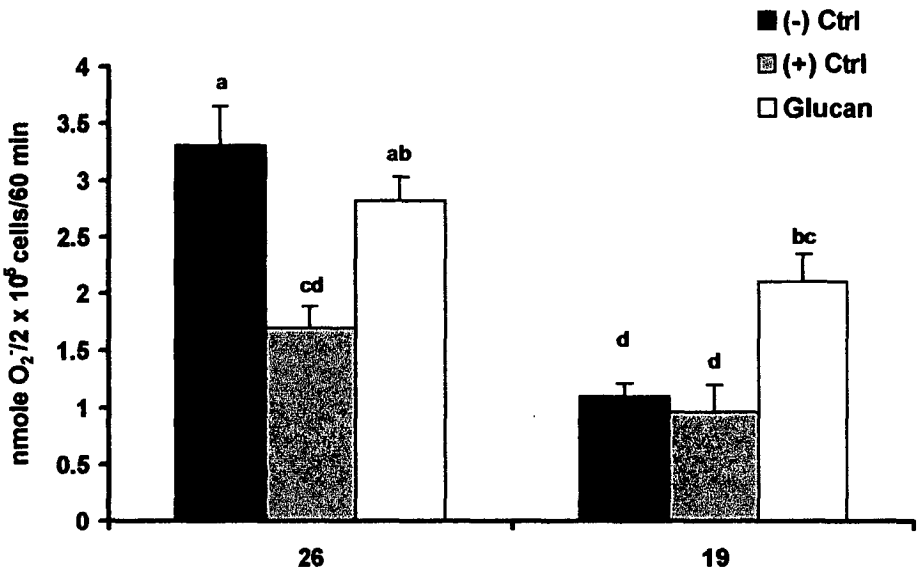


Figure 5.15 Antiprotease activity in sand whiting held at **a) 26 °C** and **b) 17 °C**. Bars are means ± s.e.; common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

a)



b)

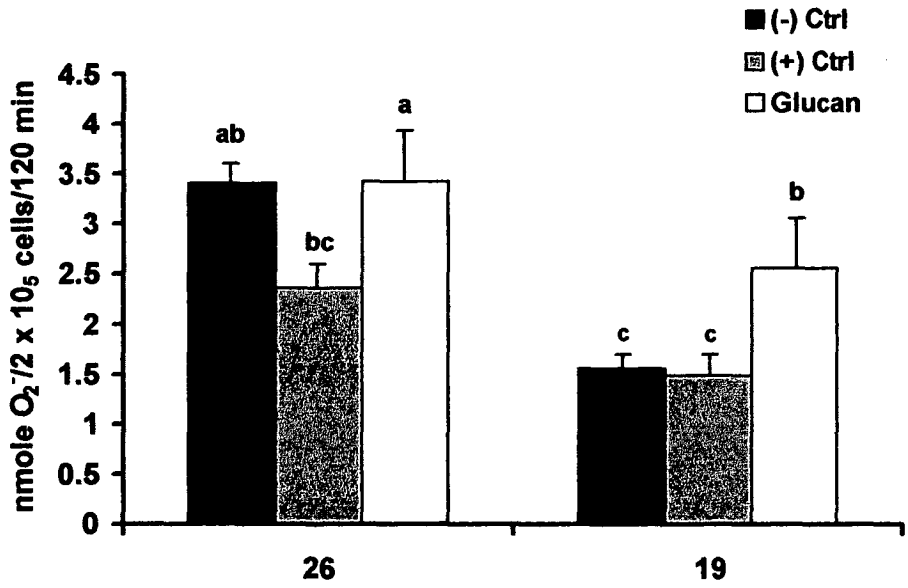


Figure 5.16 Extracellular O_2^- production in sand whiting after **a) 60 min** and **120 min** incubation. Bars are means ± s.e. common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

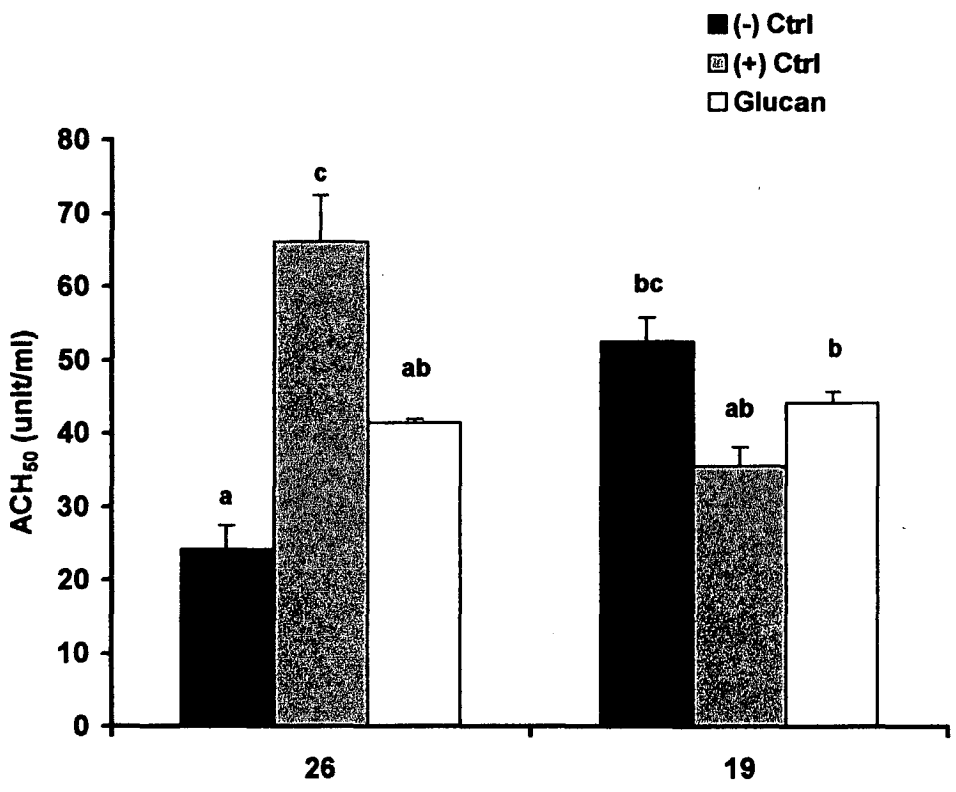


Figure 5.17 Alternative complement activity in sand whiting. Bars are means ± s.e.; common letters not significantly different by Tukey-Kramer HSD test at P=<0.05.

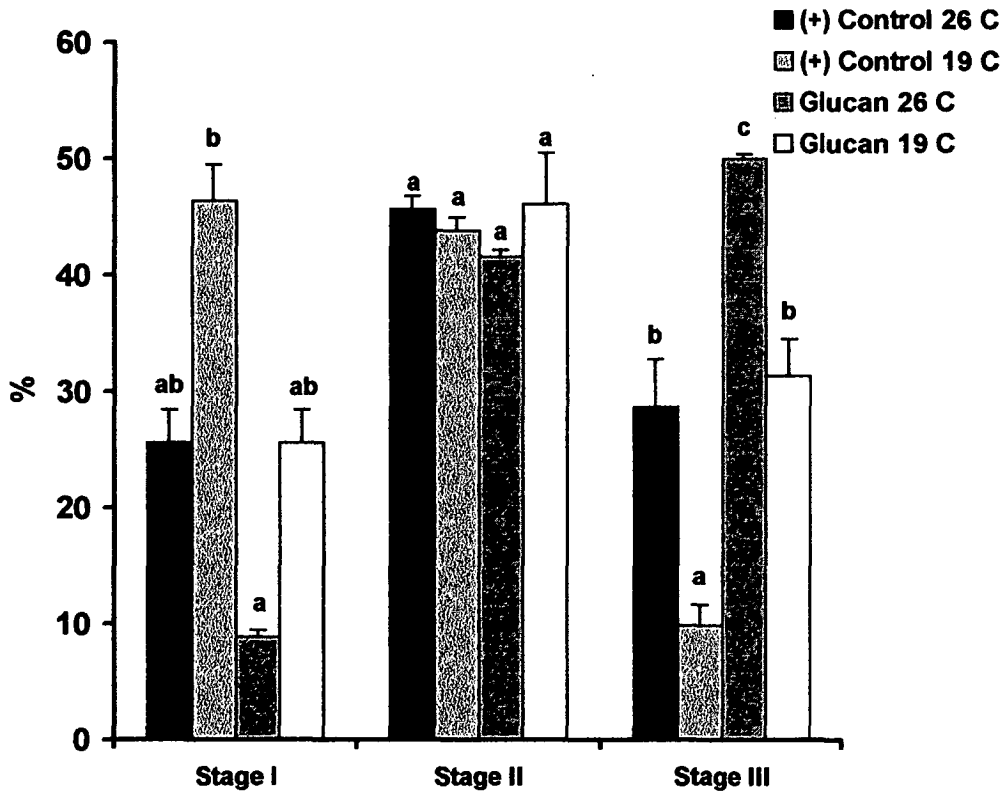


Figure 5.18 Differential granuloma counts in sand whiting. Bars are means ± s.e.; common letters within same stage or group not significantly different by Tukey-Kramer HSD test at P=<0.05.

5.4 DISCUSSION

5.4.1 Effects of fish oil supplementation in experimental EUS

Some studies have reported that the addition of polyunsaturated fat, present in fish oil, to fish diets can ameliorate the immunosuppressive effects of low temperature (Blazer 1991; Lingenfelser *et al.* 1995). This is due to the maintenance of biological membrane fluidity and function resulting from an increase in the unsaturated to saturated fatty acids ratio, known as homeoviscous adaptation (Cossins 1977). However, the results of the present study showed that the addition of fish oil in the diet failed to enhance the inflammatory response of *A. invadans*-infected sand whiting subjected to decreasing temperature. Based on the granuloma counts and the intensity of cellular infiltration, there was no significant difference in the response of EUS-affected fish fed with low fish oil diet or high fish oil diet and held at low temperature. In some instances, the fish fed with low fish oil diet even showed higher granuloma counts and greater inflammatory cell infiltration than fish fed with high fish oil diet. Fish oil supplementation was also inadequate to prevent some mortality incurred in fish stressed by rapid temperature drop.

The influence of dietary lipids in fish immunity is not very well understood due mostly to contradictory results as reported by different authors (Fletcher 1997). Sheldon and Blazer (1991) and Lingenfelser *et al.* (1995) both reported enhanced macrophage function and disease resistance in channel catfish fed supplemented with n-3 fatty acids which is generally associated with fish oil. Diets enriched with fish oil had also been reported to improve immunocompetence in rainbow trout (Kiron *et al.*1995), carp (Pilarczyk 1995) and in gilthead sea bream (Tort *et al.* 1996). However, Erdal *et al.* (1991) reported that dietary inclusion of omega-3 fatty acids caused significant decrease in survival and antibody levels in Atlantic salmon with concomitant degenerative changes. Moreover, Fracalossi and Lovell (1994) and Fracalossi *et al.* (1994) also reported the immunosuppressive effects of menhaden oil on the survival of channel catfish when challenged with *Edwardsiella ictaluri.*

Studies on the effects of dietary n-3 fatty acids on mammals consistently produced immunosuppression of the cellular immune response due to decreased lymphocyte proliferation (Yaqoob *et al.*1994), reduced lymphocyte adhesion to macrophages (Sanderson and Calder 1998), inhibition of the antigen-presenting function of mononuclear phagocytes (Hughes and Pinder 1997) and selective effects on the production of various inflammatory mediators (Yaqoob and Calder 1995).

High consumption of n-3 polyunsaturated fatty acids (PUFA) apparently results in a decrease in arachidonic acid levels (n-6 PUFA) and an increase in eicosapentanoic acid (EPA) and docosahexanoic acid (DHA)(Calder 1996). Since EPA and DHA could competitively inhibit the conversion of arachidonic acid to prostaglandins (PG's) and leukotrienes (4-series LT's), such condition could lead to elevated production of EPA-derived eicosanoids (such as PG_3 and LTA_5) which are less biologically potent as immune regulator and inflammatory mediator than arachidonic acid-derived eicosanoids. It is likely that these mechanisms could explain the reduced inflammatory and granulomatous response exhibited by EUS-affected sand whiting fed with high fish oil diet. This observation warrants further investigation in view of the trend to develop high oil diets for use in aquaculture. Waagbo (1994) suggested that the role of dietary lipids in fish to maintain membrane fluidity at low temperature and its immunosuppressive effects on cellular immunity needs cautious evaluation.

5.4.2 Effects of L-cysteine ethyl ester on epidermal mucus

L-cysteine ethyl ester (L-CEE), a mucolytic agent, belongs to a class of therapeutic drugs, which can reduce the viscosity of mucus by cleaving the disulfide bonds of mucopolysaccharides (Yang and Albright 1994). The same study reported the therapeutic effect of L-CEE in coho salmon, *Onchorynchus kisutch*, against the accumulation of the diatom, *Chaetoceros concavicornis*, in the gills due to excessive mucus production. The L-CEE treatment produced less viscous mucus that was readily washed off from the gills, thus preventing the build-up of the diatoms in the thick mucus.

Histopathological examination showed that only one fish from the L-CEE treatment had bacterial and fungal elements attached on the epidermis. On the other hand, 8 fish (3 held at 26 °C and 5 fish at 19 °C) from the positive control (fish exposed to fungal mats alone) and 3 fish (2 at 26 °C and 1 at 19 °C) from the cohabitation treatment had bacterial and/or fungal elements on their skin. It appears that L-CEE treatment prevented the attachment of microbial elements on the skin of the experimental fish but because of the additional stress effects of salinity on sand whiting and the heavy mortality in the experiment, the quantitative data did not show any significant difference between the treatments and further investigations are required to assess the effectiveness of this therapeutant against *A. invadans* attachment on the skin. Wood *et al.* (1988) reported that in brown trout, *Salmo trutta*, spores of the pathogenic *Saprolegnia diclina-parasitica* could attach more rapidly on the external mucus than its saprophytic species. In addition, greater numbers of viable propagules of the pathogen were retained on the skin compared with the saprophytic species. Hence, measures that will result in the continuous sloughing of epidermal mucus could alleviate the problem of fungal spore attachment.

5.4.3. Effects of levamisole and yeast glucan

Levamisole and yeast glucans were used in this study because of their stimulatory effects on the innate components of the immune system. Levamisole, an antihelminthic drug, is a known T-lymphocyte and macrophage stimulant (Siwicki 1989; Kajita *et al.* 1990) while yeast glucans were reported to enhance fish resistance against infection due to its effects on macrophages(Sakai 1999). Since low and/or fluctuating temperatures were shown to reduce the response of fish against *A. invadans*, the use of these substances could alleviate this problem.

5.4.3.1 Haematological parameters

Hematocrit level of EUS-affected fish was lower, although not significantly, than the (-) control sand whiting at 26 °C. This had been reported in snakehead, *Ophicephalus striatus*, with advanced EUS lesions (Cruz-Lacierda and Shariff

1994) and in snakeskin gourami, *Trichogaster pectoralis* in Sri Lanka (Pathiratne *et al.* 1999). Cruz-Lacierda and Shariff (1994) suggested that this could be due to low hematopoiesis and physiological dysfunction of the severely-affected spleen and kidney. The higher hematocrit levels in immunostimulated fish, although not significant in levamisole-treated group, signify that these substances may also aid in maintaining the general health of infected fish. However, at low temperature, there was no significant difference in the hematocrit levels of (+) fish and the levamisole- and glucan-treated fish.

The relatively higher hematocrit in sand whiting kept at 17 °C may not be the direct effects of temperature. Low hematocrit levels at low temperature has been reported in field populations of striped mullet, *Mugil cephalus* (Cameron 1970) and goldfish, *Carassius auratus*, (Munkittrick and Leatherland 1983). However, there were also reports of high hematocrit at low temperatures in field populations of pinfish, *Lagodon rhomboides*, (Cameron 1970), in commercially-bred goldfish (Minkittrick and Leatherland 1983) and in rainbow trout subjected to simulated "winter" temperatures (Houston *et al.* 1996). It has been reported that seasonal variations in the hematocrit of rainbow trout were not due to temperature changes and probable causes such as metabolic adaptations and activity and even diet were proposed. (Denton and Yousef 1975).

As for the differential leukocyte counts, the PMN did not vary significantly between treatments but (+) control fish had higher counts than the (-) control probably as a response to the infection. At 26 °C, levamisole- and glucan- treated fish had higher PMN counts than the (+) control. Since blood samples were collected at day 8 post infection, the PMN counts were relatively low as these cells are more prominent in acute infections.

EUS-affected fish at 26 °C exhibited lymphocytopenia. Similar response was also reported by Cruz-Lacierda and Shariff (1994) in snakehead (*O. striatus*) during the early stage of EUS. Higher lymphocyte counts were found in glucan- and levamisole-treated fish but were not significantly different from the nonstimulated, infected fish. However, lymphocytopenia was compensated by an increase in the monocytes of fish kept at 26 °C. At 17 °C, monocytes remained

low but lymphocytes were higher than in fish at 26 °C. It is possible that low temperature does not affect the release of lymphocytes from the hematopoeitic tissues but it is likely that their immune functions, such as cytokine production may be affected. It is also likely that the release of inflammatory mediators was affected due to the low monocyte counts in fish held at 17 °C. This probably explains the delayed granuloma formation since epithelioid cells usually originate from tissue macrophages and monocytes. The administration of yeast glucans and levamisole did not significantly enhance the migration and circulation of monocytes in fish at 17 °C.

5.4.3.2 Nonspecific immune responses

Some components of the nonspecific humoral immunity include lysozyme antiprotease factors and the alternative complement pathway. In this study, the lysozyme levels in sand whiting did not vary significantly between treatments. However, it appears to be sensitive to low temperature since the untreated control fish at 26 °C showed higher lysozyme level than the untreated control fish at 17 °C. This was also reported in other species such as plaice, *Pleuronectes platessa*, (Fletcher and White 1976), carp, *Cyprinus carpio*, (Studnicka *et al.* 1986), and dab, *Limanda limanda* (Hutchinson and Manning 1996).

In fish, lysozyme is mainly found in leukocyte-rich tissues, such as head kidney, skin, gills and the digestive tract, and primarily acts as an antibacterial since it can break the peptidoglycan layers of Gram-positive bacteria and may lyse Gram-negative bacteria in the absence of complement (Yano 1996). Thus, it could be used as an index of the nonspecific immune capacity in fish.

Results from this study showed that levamisole-treated fish exhibited higher lysozyme activity than the infected control and glucan-treated fish at 26 °C and 17 °C. In carp fed with levamisole-supplemented diet, lysozyme activity increased 2 weeks after the last administration and then peaked 4-8 weeks after the treatment (Siwicki 1989). However, in this trial, lysozyme activity was determined 3 weeks after the last treatment and therefore it is possible that the

peak lysozyme activity was not detected. Glucan administration failed to enhance the nonspecific humoral response of EUS-affected sand whiting at 26 °C and 17 °C in this trials.

Antiprotease activity is a measure of the capacity of fish to neutralise extracellular proteases released by most bacteria during infection thus extensive tissue damage could be prevented (Alexander and Ingram 1992). Antiproteases include α_2-macroglobulin, α_2-antiplasmin and α_1-antitrypsin which act as acute phase proteins and usually increase in the serum and mucus during infection or post-immunisation (Thompson *et al.* 1994). Results from these trials show that this humoral factor was inhibited at 17 °C since generally, most enzymatic reactions are usually slowed down by low temperatures. Antiprotease activity was high in *A. invadans* infected fish and was significantly enhanced by levamisole at 26 °C and at 17 °C and to some extent, but not significantly, by glucan treatment.

The alternative complement pathway (ACP) and the classical complement pathway (CCP) constitute the complement system in fish, as in mammals, and the system is made up of a series of protein components. Activation of either pathway usually lead to the production of biologically active factors that are involved in inflammatory responses (Yano 1996). In the ACP, the third complement component, C3, is directly activated in the presence of Mg^{++} by substances such as the lipopolysaccharide of Gram-negative bacteria, inulin zymosan and rabbit erythrocytes. In the assay used in these trials, the rabbit erythrocytes were the activating factor and at the same time the target cells with the inclusion of Mg^{++} in the EGTA buffer. The addition of EGTA, a Ca^{++} chelator will inhibit the CCP since this pathway requires both Mg^{++} and Ca^{++} (Matsuyama *et al.* 1988). Complement can either lyse pathogens directly or through enhanced phagocytosis facilitated through the opsonisation by C3b and the chemotactic action of C5b and eventual degradation of the pathogen (Yano 1996).

The ACP activity of sand whiting showed that it is more active at 19 °C than at 26 °C. This result concurs with reports from other fish species with optimum ACP activity at low temperature (Matsuyama *et al.* 1988; Yano 1996). Since antibody production is reduced at low temperature and it is required for the

activation of the classical pathway, the ACP would be a substitute defense mechanism.

At 26 °C, *A. invadans*-infected fish showed an increase in ACP activity but was lower at 19 °C and in glucan-treated fish. In Atlantic salmon, *Salmo salar*, infected with *Vibrio salmonicida*, nonvaccinated and vaccinated fish exhibited a decreased C3 activity (C3 component common to CCP and ACP) due to consumption of the factor, as in septic conditions in human patients (Brattgjerd *et al.* manuscript for publication). This could possibly explain the lower ACP activity in EUS affected fish at 19 °C. In mammals, macrophages are involved in C3 synthesis (Brattgjerd *et al.* manuscript for publication), thus it is also likely that the low C3 production was due to the low macrophage population in sand whiting at low temperature, as shown in the differential leukocyte counts in Experiments I.B.

Aside from humoral factors, fish also rely on nonspecific cellular immune mechanisms against pathogens. One of the most important mechanisms is the process of phagocytosis wherein phagocytes such as neutrophils and macrophages ingest and degrade invading pathogens. The process is composed of three main phases: attachment of the particle to the cell surface, ingestion involving the formation of a phagosome, and the breakdown of the particle within the phagosome. According to Blazer (1991), phagocytosis is not affected by any temperature acclimation but the pathogen killing mechanism is suppressed by low temperatures. The likely explanation is that phagocytosis only relies on membrane fluidity while degradation or killing of the pathogen is controlled by enzymatic reactions. This was confirmed in the present study since there was no significant difference in the phagocytic activity in sand whiting held at either 26 °C or 19 °C.

Diet supplementation with either levamisole or glucans only slightly enhanced phagocytosis in EUS-affected fish 3 weeks after the administration. Siwicki (1989) reported higher phagocytic index in carp, *Cyprinus carpio*, 2 weeks after the last levamisole feeding and peaked at 8 weeks after, if fish were held at 12 °C and the stimulation lasted for 3 months. Hence, it is probable that peak

phagocytosis was likewise not detected in this experiment. Apparently, the efficacy of glucan preparations is rapid and short-lived, usually lasting 14 days (Anderson and Siwicki 1994; Galeotti 1998). Since the fish in this experiments were sampled 3 weeks after the last glucan treatment, it is possible that its stimulatory effect had already declined.

In Experiments II.A and II.B, the samples for the extracellular production of O_2^- and ACP were obtained 8 days after the last glucan feeding. Production of reactive oxygen species (ROS), one of the pathogen-killing mechanisms employed by macrophages (Secombes 1996), was lower at 19 °C than at 26 °C in sand whiting. Hardie *et al.* (1994) showed that ROS production in rainbow trout is low at lower temperatures the production of macrophage activating factor from T-lymphocytes was suppressed. However, results showed that yeast glucans significantly enhanced the respiratory burst of sand whiting with *A. invadans* infection at 26 °C and at 19 °C. It has been shown by Engstad and Robertsen (1994) that Atlantic salmon macrophages possess receptors for glucans derived from yeast and mycelial fungi. This could explain the stimulatory effect of glucan preparations on macrophages. These results are consistent with the quantitative analysis of the mycotic granulomas. Stage III granulomas, which show lysed fungal cell walls, were significantly higher in glucan-treated fish than the (+) control fish while Stage I granulomas were higher in number in (+) control fish held at 19 °C. Moreover, it appears that the yeast glucan treatment conferred some degree of protection since mortality was higher in unstimulated, infected fish than in glucan-treated fish,

Taken all together, the results show that yeast glucans and levamisole have potential prophylactic use against EUS during risk periods, such as when temperature decreases, but further investigations are required, especially on the dosage, timing and route of application and the duration of efficacy or stimulatory effect of the substances. The use of L-CEE also requires further studies to assess its impact on the epidermal mucus coat and identification of other prophylactic measures against EUS is still warranted.

Chapter Six

Overview

6.1 General Discussion and Conclusions

The involvement of various biological and environmental factors in EUS outbreaks made the pathogenesis of the disease difficult to elucidate when it was first reported in Southeast Asia during the early 1980's. In the Philippines, EUS typically occurs during the cold months, characterised by decreasing and fluctuating water temperature, and usually preceded by rainfall events. For this reason, the influence of low and/or fluctuating temperature on the pathogenesis of EUS was investigated in this study, and eventually the efficacy of some prophylactic agents to ameliorate the effects of temperature in fish susceptible to *A. invadans* infection were trialled.

EUS lesions were experimentally induced in two fish species, three-spot gouramis and sand whiting, by intramuscular injection of *A. invadans* zoospores with very minimal mortality in both species. The sequential pathology of the infection was established for each species which was mainly characterised by the formation of mycotic granulomas and the infiltration of inflammatory cell in the affected tissues. Quantification of mycotic granulomas and the extent of cellular infiltration in a sampled area showed that three-spot gouramis manifested a more vigorous granulomatous and inflammatory response than sand whiting. This indicates that response to *A. invadans* invasion is host species specific. The degree of difference in terms of the cellular response could possibly explain the capacity of some species to survive with complete resolution of the lesions while high mortality is incurred in other EUS-susceptible species.

The EUS pathology model developed in the preliminary experiments was used to examine the effects of either rapid or gradual temperature drop in sand whiting. Based on the sequential qualitative and quantitative histopathology, fish subjected to temperature manipulations and subsequently held at 17 °C showed delayed formation of mycotic granulomas and reduced influx of inflammatory cells to the affected area while fish kept at 26 °C exhibited intense granulomatous and inflammatory response. Consequently, the extent of tissue damage was greater and the resolution of lesions took longer in fish held at low temperature than in fish maintained at high temperature. In the natural environment, such condition

could lead to fish mortality not only because of *A. invadans* infection but also due to secondary infections. The down-regulation of the inflammatory process by low temperature can be explained by the inability of T-lymphocytes to adapt to such conditions, therefore their immune functions, like production of cytokines that could initiate the inflammation and activate macrophages, are affected. In addition, concentrations of stress hormones, such as glucocorticoids, could increase due to temperature stress and thus, can also influence the immune response of fish.

Temperature manipulations and exposure to *A. invadans* elements at low salinity failed to initiate any change in the epidermis and mucous cell concentrations in sand whiting, hence no EUS lesions were produced. However, the additional osmotic stress imposed on the fish, which are normally marine or estuarine, during the experiment apparently precipitated high mortality, and therefore, no definite conclusions can be drawn from the experimental results. Histopathological examination, nevertheless, showed epidermal spongiosis and oedema, which could be associated with salinity stress. It is possible that this epidermal response contributes to the susceptibility of estuarine fish to *A. invadans* invasion since previous reports suggested the association of low salinity with EUS outbreaks in this species.

EUS lesions were induced in skin-abraded three-spot gouramis which were exposed to *A. invadans* zoospores. The fish were more likely to get infected when kept at 19 °C than at 26 °C since abraded areas heal rapidly at higher temperature. Daily temperature fluctuations led to higher infection rate and mortality than when the experimental fish were held at stable 26 °C. Histopathological examination showed intense mycotic granuloma formation and early onset of repair in fish held at 26 °C, while fish subjected to daily temperature variations showed reduced granuloma formation with extensive sarcolysis and oedematous response. The pathogenicity of *A. invadans* apparently increased after a single passage in a fish and the use of pond water improved the induction of EUS in experimental fish

Quantitative examination of the intact skin of three-spot gouramis subjected to rapid temperature drop and subsequently maintained at 19 °C did not show any significant change in the epidermal thickness and the distribution of mucous cells. The temporal trends , in terms of the mucous cell counts and epidermal thickness, indicate that the fish's skin could recover from physical and temperature stress after 4 days, preventing the invasion of *A. invadans*. Histochemical analysis of the skin of fish subjected to daily fluctuations of temperature showed a decreasing trend in the total mucous cell counts due to significant decrease in PAS (+) cells (neutral mucopolysaccharide) and the decreasing trend in the AB(+) cells (acidic mucopolysaccharides) during the 24-day experiment. Even so, these changes were insufficient for the attachment of *A. invadans* on the skin of three-spot gouramis. However, it is possible that stress caused by handling and temperature manipulation in the experiment could have eventually caused mucus depletion, especially in areas with less mucous cells, which could be vulnerable sites for infection.

Possibly, more useful information could be obtained by exploring lectin/receptor interactions. According to Danguy *et al.* (1991), histochemical methods have limited specificity to demonstrate polysaccharide moieties and suggested the use of lectins as histochemical probes. In this context, Bondad-Reantaso *et al.* (1999) and Sanpei *et al.* (1999) recently reported a D-galactose-binding hemagglutinin in *Aphanomyces* isolates from Japan, Philippines and Bangladesh, which could be important in the pathogenesis of EUS. This aspect certainly warrants further investigations in order to elucidate the significance of such interactions in the pathogenesis of EUS.

The effect of fish oil supplementation was examined in *A. invadans*-infected sand whiting based on sequential histopathological signs. Results showed that the addition of fish oil in the diet did not ameliorate the down-regulation of the inflammatory and granulomatous response caused by decreasing and low temperature. The presence of high n-3 fatty acids (EPA and DHA) in fish oil competitively inhibits the production of arachidonic acid-derived immune and inflammatory mediators, hence, the suppressed response in sand whiting against *A. invadans* when held at low temperature.

The potential of a mucolytic agent, L-CEE, to prevent the attachment of fungal elements on the skin of sand whiting was also studied. Although results showed that the treatment seem to have inhibited pathogen attachment, further studies to assess its effectiveness are required, moreover, prophylactics which could enhance the protective capacity of the skin should be identified and trialled.

Based on the haematological and nonspecific immune parameters measured in this study, low temperature produced a monocytopenia in EUS-affected fish. Phagocytosis was not affected by low temperature but the extracellular production of superoxide anion was inhibited. Lysozyme and antiprotease activities in sand whiting were likewise inhibited at 17 °C but the alternative complement activity remained high at low temperature. It is possible that this is a mechanism for the fish to minimise tissue damage by just using a more effective method rather than producing different factors which may not be capable of killing the pathogen.

Treatment with yeast glucans alleviated inhibition of the respiratory burst due to *A. invadans* infection at 26 °C and at 19 °C, however, it did not exhibit any stimulatory effects on nonspecific humoral responses while levamisole seemed to have stimulatory effects on both lysozyme and antiprotease activities. These results showed that these two immunomodulators have some potential use as preventive measures against EUS. However, as suggested in Chapter 5, more studies on the efficacy of these substances are still needed. It might be important to mention that the yeast glucan used in this study (Aquagard) was used last year in EUS-affected silver perch in New South Wales and it apparently worked (R. Callinan, pers. comm.). Nevertheless, the use of such prophylactics must always be accompanied by proper pond management and husbandry to optimise their value.

Overall, the research documented in this thesis has provided useful insights into the pathogenesis and control of EUS. However, the hypothesis that low and/or fluctuating temperatures, as observed in association with EUS outbreaks in the Philippines, are a sufficient cause was not proven and the key inciting factors

related to outbreaks of the disease in that country remain to be identified. Nevertheless, recent findings by Kanchanakhan *et al.* (1999) that a virus and *A. invadans* at 20 °C induced EUS but not with the same combination at 29 °C suggests that low temperatures may be a sufficient cause in concert with another sufficient cause. Figure 6.1 illustrates a likely sufficient cause for EUS, with virus and temperature as possible predisposing factors to *A. invadans* infection. That this may apply to the Philippines is emphasised by the findings of Lio-Po *et al.* (1999), who isolated a virus from catfish cohabited with snakeheads with EUS in that country. Also, low water temperatures play a part in exacerbating the effects of EUS once it is established by perturbing the immune response.

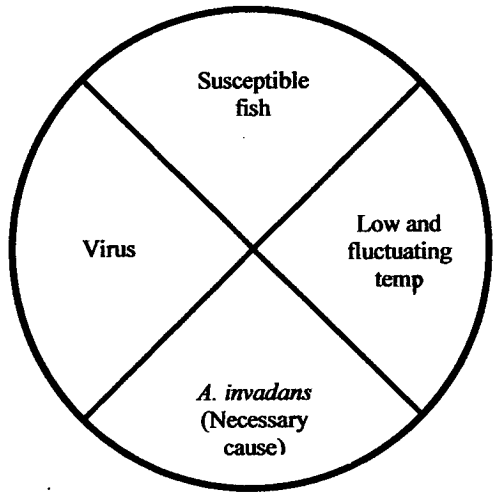


Figure 6.1 Schematic representation of a sufficient cause for EUS with combination of virus and low and/or fluctuating temperature as factors predisposing susceptible fish to *A. invadans* infection.

6.2 Scope for further investigations

The following five main areas warrant further research. Some specific topics were mentioned in the previous section, but will be reiterated here.

1. Sufficient cause of EUS in the Philippines. Trials need to be performed to induce EUS to fully understand the combined effects of viral pathogens (EUS-related viruses) and low and/or fluctuating water temperatures, in the presence of *A. invadans* isolates. The results could possibly explain the occurrence of EUS in the Philippines wherein water temperature seems to be the only environmental factor consistently associated with the outbreaks of the disease and the presence of viral pathogens in some EUS-affected fish.

2. Role of other environmental and biological factors in EUS pathogenesis. The possible effects of decreasing salinity, specifically on the epidermis of susceptible fish, also require further study. This aspect was not fully investigated in this research. It is likely that salinity could be a sufficient cause for EUS among estuarine fish in the Philippines, since pH seems to be unrelated to such outbreaks. However, low pH and acid run-off could be potential factors in EUS occurrences in other countries, thus it is also essential to assess its impact through data collected from fieldwork. Moreover, there are no data or studies undertaken yet on the involvement of algal toxins in EUS outbreaks. It would be worthy to look into this aspect in Asian EUS, since ulcerative mycosis of Atlantic menhaden had been related to a toxic dinoflagellate, *Pfiesteria piscicida*.

3. Interaction of the fungus *A. invadans* and fish using lectins as probes. Preliminary reports on the presence of a lectin (a hemagglutinin) in the fungus, *A. invadans*, warrant follow-up examination to explore the lectin/host receptor interactions, especially in the fish skin and epidermal mucus. Effects of different factors (biological and non-biological) on this interaction should also be examined as these might be significant in the pathogenesis of EUS.

4. Immune status of EUS-susceptible fish. Investigations on various immune parameters of EUS-susceptible fish, including the protective mechanisms of the

skin and epidermal mucus, should also be undertaken. These might be related with the species-specific response of susceptible fish to *A. invadans* infection.

5. Control and prophylactic treatments against EUS. The efficacy of various immunostimulants or other prophylactics could also be tested in conjunction with the above-mentioned areas. Also, other substances that could improve the protective mechanisms of the skin of susceptible fish need to be identified and trialled. Further studies on the dosage, duration and timing of application and route of administration of such prophylactics need to be undertaken, and these could be eventually trialled in field studies.

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Appendices

APPENDIX 1

Sporulation of an *Aphanomyces invadans* isolate (24P) in different liquid media

Background

This short experiment was undertaken to determine which liquid medium could induce maximum production of *Aphanomyces invadans* zoospores to be used in subsequent experimental induction of epizootic ulcerative syndrome (EUS) in susceptible fish.

Materials and Methods[1]

Agar blocks containing *A invadans* sp. hyphae were aseptically placed in Schott transparent bottles with 50 ml glucose-yeast broth (3 blocks x 5 bottles) to grow fungal mats. After 4 days, the fungal mats were washed 5x in sterile distilled water and one fungal mat was placed in a Petri dish with 20 mL of one of the following: autoclaved distilled water (3 replicates), autoclaved tap water (3 replicates), autoclaved and filtered pond water (farm dam, Glengarry, Tasmania, 3 replicates) and autoclaved sporulating medium (0.028 g $CaCl_2$ and 0.019 g KCL in 1 L distilled water, 3 replicates). After 24-hour incubation at 22 °C, the number of motile zoospores were counted using a Sedgewick-Rafter chamber under a dissecting microscope. Ten squares of the chamber were counted after loading it with 1 mL of culture fluid and three counts were made per Petri dish. Numbers of zoospores were calculated using the following formula:

$$C = \frac{n}{10} \times \frac{1000}{1}$$

where n = number of zoospores counted in 10 squares/fields

10= no. of squares randomly selected for counting

1000= total area of the chamber

1 = depth of the chamber

[1] Zoospore count technique developed by RB Callinan and GC Fraser (NSW Fisheries/Agriculture, Wollongbar Veterinary Laboratory, NSW).

Results

The following were the calculated motile zoospore counts for each medium:

	Plate 1 Count			Plate 2 Count			Plate 3 Count		
Type of medium	A	B	C	A	B	C	A	B	C
Distilled water	0	0	0	0	0	0	0	0	0
Tap water	128	115	32	165	44	6	61	117	35
Pond water	370	380	420	260	290	210	250	330	310
Sporulating medium	90	49	52	35	32	77	53	100	63

The fungal mats incubated in autoclaved pond water produced the maximum number of zoospores (31, 330 spores/mL), followed by 7,730 spores/mL in tap water, 6,530 spores/mL in sporulating medium and no motile zoospores in distilled water (most of the spores sampled from the distilled water had already encysted). The results showed that the pathogenic *A. invadans* requires some specific nutrients or salts in the medium in order to effectively sporulate. This was reported by Fraser *et al.* (1992) using fungal isolates from fish with red spot disease and Dykstra *et al.* (1986) using fungal isolates from Atlantic menhaden affected with ulcerative mycosis.

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APPENDIX 2

Preliminary studies to determine the concentration of *Aphanomyces invadans* spores for intramuscular injection in fish

Background

Reports of studies to reproduce EUS in a number of fish species were undertaken by injecting viable zoopores or by inserting small pieces of hyphae under the skin and in skeletal muscle (Hatai *et al.* 1997; Roberts *et al.* 1993; Hatai 1994; Chinabut *et al.* 1995). However, the concentrations of spores injected were either not mentioned or were probably too high relative to what actually occurs with natural infections. A preliminary study and two confirmatory studies were conducted using three-spot gourami and sand whiting to determine the zoospore concentration sufficient enough to produce EUS lesions with very minimal mortality.

Materials and Methods

Fish and acclimation procedures

Trial 1. Forty (40) three-spot gourami (mean total length: 5.88 ± 0.81 cm) were obtained from a local pet shop, randomly distributed in 4 x 40 L glass tanks with undergravel filters, and acclimated for one week.

Trial 2. Sixty (60) three-spot gourami (mean total length: 6.15 ± 0.78 cm) were randomly distributed in 6 x 40 L glass tanks and acclimated as above.

Trial 3. Seventy-two (72) sand whiting juveniles (mean total length: 7.37 ± 0.48 cm) were acclimated for 11 days in 6 x 40 L glass tanks.

The three-spot gouramis were fed with commercial fish flakes twice a day while the whiting were fed with Gibson's dry salmon pellet (1.0 mm Ø) twice daily.

One-third to one-half of the water was replaced every two days for gouramis while water exchange was done daily for the whiting. Immersion heaters were used to maintain the temperature at around 26 °C for both species. Salinity ranged from 8-10 ppt in the whiting tanks while aged municipal water (0 ppt) was used for the gouramis.

Production of *Aphanomyces invadans* zoopores

Glucose-yeast agar blocks with *A. invadans* hyphae were aseptically cut and placed in glucose-yeast broth (3 blocks x 100 ml) to produce fungal mats. After 4 days, each fungal mat was washed 5 times in autoclaved distilled water, placed in a Petri dish with autoclaved pond water and incubated at 20 – 22 °C for sporulation. An *in vitro* study (Appendix 1) showed that maximum number of spores is produced in filtered (Whatman 541 filter paper) sterile pond water, compared with sterile distilled water, sterile tap water and sporulating medium. After 24 hours, a spore estimate was made for each Petri dish using a Sedgewick-Rafter chamber under a compound microscope at x100 magnification.

Preparation of spore inoculum

Trial 1. Four concentrations of zoospores (see details under "Fish inoculation")were used to inoculate the 3-spot gouramis. From the original zoospore suspension (1.3×10^4 spores mL), serial dilutions were prepared by adding 18 mL of autoclaved pond water to 2 mL of spore suspension until 1.3×10^1 spores mL was obtained.

Trial 2. Two spore concentrations (1.1×10^4 spores mL and 1.1×10^3 spores mL) were prepared to be used as inocula for the 3-spot gouramis.

Trial 3. Two spore concentrations (1.4×10^4 spores mL and 1.4×10^3 spores mL) were prepared to be used as inocula for the sand whiting.

Fish inoculation

Trial 1. Fish from each tank (10 fish x 4 tanks) were anesthetised with 60-80 mgL^{-1} of benzocaine and injected with 0.05 mL of the following zoospore suspensions:

Tank 1	1.3×10^4 spores mL^{-1}
Tank 2	1.3×10^3 spores mL^{-1}
Tank 3	1.3×10^2 spores mL^{-1}
Tank 4	1.3×10^1 spores mL^{-1}

Trial 2. Fish were anesthetised with 60-80 mgL^{-1} of benzocaine and 30 fish (10 fish x 3 tanks) were each injected with 0.05 mL of 1.1×10^4 spores mL^{-1} suspension and the other 30 fish (10 fish x 3 tanks) were each injected with 0.05 mL of 1.1×10^3 spores mL^{-1} suspension.

Trial 3. Sand whiting (62 fish left after acclimation) were anesthetised with 50-60 $mgLl^{-1}$ benzocaine. Thirty-two fish (10 fish in one tank and 11 in two tanks) were each injected with 0.05 mL of 1.4×10^4 spores mL^{-1} suspension and 30 fish (10 fish x 3 tanks) were each injected intramuscularly with 0.05 mL of 1.4×10^3 spores mL^{-1} suspension in the dorsal trunk muscle, anterior to the base of the dorsal fin (Quadrant I, see fig. 2.1)

Fish were allowed to recover in well-aerated water before they were placed in their respective tanks.

Experimental conditions

Fish feeding was resumed a day after inoculation. The fish were observed daily for any mortality, behavioral and gross signs of fungal infection. Temperature was maintained as during acclimation period. Basic water quality parameters were also monitored during the experimental period. In all trials, the fish were euthanased with 200 mgL^{-1} benzocaine at 12 days post inoculation (p.i) for the gouramis and at 8 days p.i for the whiting.

Histopathology

Samples were fixed in 10 % neutral buffered formalin, trimmed, decalcified, processed, embedded in paraffin, sectioned at 5 μm and stained using the periodic acid-Schiff technique. Sections were examined under an Olympus BH-2 light microscope for the presence of fungal granulomas to confirm infection with *A. invadans*.

Results

Trial 1. Visible signs of fungal infection were observed at 6 days p.i. in two fish injected with the highest spore concentration. One fish had an external lesion while one fish had a slightly inflamed injection site. On day 7 p.i., one fish in tank 2 had a slightly inflamed injection site. On day 9 p.i., the lesions observed on days 6 and 7 p.i. had progressed to typical EUS lesions. Fish mortality started from day 10 p.i. until day 12 p.i when the experiment was terminated. Some of the fish in tanks 3 and 4 had pinpoint hemorrhages on the opercular area which were unlikely to be due to *A. invadans* infection. Histopathological examination showed 88.9% induction of EUS in tank 2, while 30% and 10% developed EUS in tanks 1 and 4, respectively.
(Table 1).

Table 1. Mortality and percentage of EUS induction in blue gourami injected with 4 different concentrations of *A. invadans* zoospores.

	Mortality		Histopathology		Fish with EUS (%)**
	without external lesion	with external lesion	fish without mycotic granulomas	fish with mycotic granulomas	
Tank 1(1.3×10^4)	2	1	5	2	30.0
Tank 2 (1.3×10^3)*	0	2	1	6	88.9
Tank 3 (1.3×10^2)	1	0	9	0	0
Tank 4 (1.3×10^1)	2	0	7	1	10

*Only 9 fish left since I fish did not recover after spore inoculation.

**Percentage of fish with external lesion typical of EUS plus fish with mycotic granulomas.

Trial 2. Some fish injected with 1.1 x 10^4 spores mL^{-1} in the 3 replicates had inflamed injection sites on day 5 p.i. while fish in the other treatment tanks (1.1 x 10^3 spores mL^{-1}) did not show gross signs of infection until day 7 p.i. Three fish died during the entire experimental period. There was 80-100% EUS induction in treatment 1 and 40-80% EUS induction in treatment 2 (Table 2).

Table 2. Percentage fish mortality and EUS induction in blue gourami inoculated with two different concentrations of *A. invadans* spores.

Spore Concentration (spores/mL)	Mortality (%)	Fish with mycotic granulomas (%)
1.1 x 10^4 replicate 1	0	80
1.1 x 10^4 replicate 2	20	100 (inc. mort.)
1.1 x 10^4 replicate 3	0	90
1.1 x 10^3 replicate 1	0	80
1.1 x 10^3 replicate 2	10	80 (inc. mort.)
1.1 x 10^3 replicate 3	0	40

The primary lesion manifested by 3-spot gourami can be described as necrotising myositis with dermatitis. Histopathology examination showed inflammatory cell infiltration into the epidermis, predominantly composed of lymphocytes. However, the scales remained intact. The dermis also exhibited sub-acute inflammatory response, but with focal necrotic areas. It was also invaded by fungal hyphae enclosed in layers of epithelioid cells. The necrotic muscle fibers were likewise heavily infiltrated with inflammatory cells, the infiltrate mainly made up of lymphocytes and macrophages and usually running along myotomal septa. Fungal granulomas and fibrous tissue virtually replaced the necrotic muscle. In some fish, new blood vessels, presumably capillaries, were present in the fibrous tissue surrounding the granulomas. Some fish developed lesions with minimal damage in the muscle fibers, with the collapsed fungal hyphae surrounded by thick layers of epithelioid cells. Some granulomas have cellular and fungal debris in the center.

Trial 3. No fish mortality occurred in both treatments during the trial. External signs were observed as early as day 2 p.i. in some fish in treatment 1 (1.4×10^4 spores mL^{-1}). These include loss of scales and inflammation at the injection site and its periphery. At day 7 p.i., most of the fish in the three replicates for treatment 1 had external lesions, while the fish in treatment 2 (1.4×10^3 spores mL^{-1}) had highly inflamed injection sites. For treatment 1, there was 100% EUS induction, and 90-100% for treatment 2 (Table 3).

Table 3. Percentage EUS induction in sand whiting juveniles inoculated with two different concentrations of *A. invadans* zoospores.

Spore Concentration (spores/mL)	Fish with mycotic granulomas (%)
1.4×10^4 replicate 1	100
1.4×10^4 replicate 2	100
1.4×10^4 replicate 3	100
1.4×10^3 replicate 1	100
1.4×10^3 replicate 2	100
1.4×10^3 replicate 3	90

As in the 3-spot gouramis, the lesions in sand whiting were consistent with those described from EUS-affected fish. In most lesions, the epidermis was infiltrated with macrophages and lymphocytes. The scales appeared intact but in some sections, especially those from treatment 1, the epidermis and scales over the lesions were lost. The dermis was oedematous and highly inflamed with mycotic granulomas and necrotic areas.

The skeletal muscles were likewise oedematous and markedly infiltrated with inflammatory cells, usually tracking down along myotomal septa, from the epaxial to the hypaxial muscles. There was extensive myonecrosis mycotic granuloma formation. Fungal hyphae were usually enclosed in 4-5 layers of epithelioid cells. Mycotic granulomas were also observed in the kidney and spinal cord, and sometimes in the spleen and liver.

Discussion

Results from these trials showed that EUS lesions can be induced in 3-spot gourami and sand whiting by intramuscular injections of zoospores in concentrations much less than those which were used in previous studies. Approximately 55- 70 spores/fish produced the pathological signs of EUS with very minimal mortality in 3-spot gourami and no mortality in sand whiting. Hatai (1994) injected goldfish with 0.2 mL suspension of 2.5×10^4 spores mL^{-1} (5000 spores).

Although the data from trial 1 were not conclusive because of lack of replication, the two confirmatory experiments showed an average of 90% induction in 3-spot gourami injected with 0.05 mL of 1.1×10^4 spores mL^{-1} suspension and 67% in fish injected with 0.05 mL of 1.1×10^3 spores mL^{-1} suspension. In sand whiting, EUS lesions were produced 100% and 97% in fish injected with 0.05 mL of 1.4×10^4 spores mLl^{-1} and 1.4×10^3 spores mLl^{-1}, respectively. Singhal et al. (1987) reported 95-100% reproduction of saprolegniasis through intramuscular injection, compared with 75-100% induction through scale removal. EUS induction of 30-80% was previously obtained in abraded 3-spot gourami (Catap, unpub. data).

The main histopathological features of EUS, chronic granulomatous response and extensive infiltration of inflammatory cells, elicited by *A. invadans* were observed in both species of fish. Hence, this method can be used with these two species to establish a model of EUS pathology and the effects of factors reported to be associated with EUS outbreaks can be studied using this model.

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APPENDIX 3

Preliminary trial on the effects of temperature on the epidermis and density of mucous cells in sand whiting, *Sillago ciliata* Cuvier

BACKGROUND

This trial was undertaken to investigate if gradual or rapid drop in temperature could induce epidermal changes in sand whiting, a fish species susceptible to *Aphanomyces invadans* infection. Studies to induce *Saprolegnia* infection in channel catfish and in rainbow trout have used combinations of abrasion stress and temperature manipulations prior to spore exposures (Howe and Stehly 1998; Howe *et al.* 1998). In another report, saprolegniasis was induced in coho salmon using a "net-shaking" stress method to induce epidermal damage and then exposed to spores (Kubota undated). For this preliminary trial, the "net-shaking" stress method was used in combination with temperature manipulations in an attempt to induce EUS in sand whiting.

MATERIALS AND METHODS

Fish and acclimation procedures

One hundred and forty-four (144) sand whiting fish (mean weight 9.4 ± 1.9 g) were randomly distributed in 40-L glass tanks (8 fish x 18 tanks) with undergravel filters and acclimated for 25 days. The fish were fed with Gibson's dry salmon pellet (2.0 mm Ø) daily at 2.0 % body weight. One third of the water in all tanks was replaced every 3 days. During acclimation, the salinity of the water used for water exchange was gradually decreased from 8 ppt to 0 (freshwater) by increasing the proportion of dechlorinated municipal water to the seawater in the water-holding tank. Immersion heaters were used to maintain the temperature at the desired level. Mean daily temperature was 26.0 ± 0.38 °C and mean daily dissolved oxygen was 6.1 ± 0.58 mgL^{-1}. The pH ranged from 5.6-7.4 and NH_3-N from 0.1-0.25 mgL^{-1} during the acclimation period.

Experimental design

After acclimation, the sand whiting were allocated into 3 treatment groups. Six tanks of fish served as control. For this group, fish in 2 tanks were maintained at 26 °C, another 2 tanks of fish were subjected to gradual decrease in temperature and the other 2 tanks of fish were subjected to rapid decrease in temperature. There was no *A. invadans* spore exposure for this group. Similar temperature treatments were used for the second group (S-group) and third group (S + S group). However, after the drop in temperature (gradual or rapid), the fish in the S-group were exposed to *A. invadans* spores while the fish in the S + S group were swayed in air in a scoop net for 30 sec before exposure to zoospores.

All treatments were done in two replicates. For the gradual temperature drop, immersion heaters were adjusted to lower the temperature to at least 19 °C over a period of 7 days. For the rapid temperature drop, the acclimation temperature was lowered to 19 °C over a period of 24 hours.

Production of *A. invadans* mats and spores

Methods to produce mats and to sporulate *A. invadans* were as described in Chapter Two. In addition to spore production, fungal mats were produced to be placed in *A. invadans*-treated tanks. Each fungal mat was washed 5 times in autoclaved distilled water, placed in a histocassette and then distributed in the treatment tanks (2 histocassettes per tank). It was assumed that the mats would sporulate after 18-24 hours, thus ensuring exposure of fish to spores after the initial exposure.

Experimental treatments and sampling procedures

No feeding was made a day before the fish were stressed and exposed to fungal spores. After water temperature was dropped from 26 °C to 19 °C, fish in each replicate treatment tanks (S-group) were exposed to *A. invadans* by transferring the fish in 4-L glass beakers with autoclaved pond water containing 25,000 spores L^{-1} concentration for 30 min. For the S + S group, the fish were swayed in air in scoop net at 180° angle for 30 sec before exposure to the same concentration of spores for 30 min.

The fish were returned to their respective tanks after the spore exposure treatment. Temperature was maintained at 19 °C in tanks subjected to temperature drop and feeding was recommenced 24 hours after the treatment procedures, feeding rate was decreased to 1.0% body weight for fish at low temperature. Temperature and DO levels were monitored during the experiment and water exchanges were done as during acclimation period.

Fungal mats in histocassettes were placed in the treatment tanks at day 2 post exposure (p.e.). Samples for histopathology were obtained 24 hrs after initial exposure to fungal zoospores and then at day 3 (24 hours after fungal mats were placed in tanks),day 6 and day 9 p.e. Fish were sacrificed by hitting their head with the wooden handle of a laboratory spatula and were then transversely cut in half. The anterior sections (with the head) were immediately fixed in 2.5 % cacodylate-buffered glutaraldehyde with 2% Alcian blue to stain the mucus coat (Powell *et al.* 1992).

Histopathology

After 24-hour fixation, a small portion of the dorsal muscle, just anterior to the dorsal fin was carefully sliced with a sharp, single-edge blade. and coated with 10% gelatine. The coated samples were further fixed for another 24 hours. The tissue samples were then washed with cacodylate buffer and transferred to 70% ethyl alcohol. The JB4 resin embedding kit was used instead of paraffin. Prior to embedding, the samples were transferred to 95% ethyl alcohol, left overnight in a shaker for infiltration. Sections were embedded in Beem embedding capsules with resin mixture prepared as described in the embedding kit instructions. The resin polymerized after 30-45 min and samples trimmed prior to sectioning. Sections (4-5 μm) were cut using glass knives. Sections were stained with periodic acid - Schiff (PAS) and hematoxylin-eosin (H & E).

Using an Olympus BH-2 light microscope with a micrometer eyepiece, 10 random measurements of the epidermal thickness were taken from one scale unit of each sample at x400 magnification. Mucous cells were counted in 250 μm length of epidermis in one scale unit per sample.

Statistical analysis

Epidermal thickness measurements and mucous cell counts were subjected to normality and homogeneity of variance tests. Mucous cell counts were square root transformed prior to a full factorial three-way ANOVA to detect difference between treatments.

RESULTS AND DISCUSSION

High fish mortality occurred during the acclimation and the experimental period (Table 1). All fish in the S+S groups held at 26 °C or subjected to rapid temperature drop died 24 hours after they were stressed and exposed to fungal spores, hence no data were obtained for statistical analysis. It is likely that sand whiting were more sensitive to the stress method used in this experiment compared to coho salmon and Asian sea bass (*Lates calcarifer*, commonly known as barramundi in Australia) as reported in previous studies. In addition, it seems that the sand whiting could not tolerate long periods in freshwater (0 ppt) as mortality in most tanks occurred even during the acclimation period.

Statistical analysis showed that there was no significant change in either the epidermal thickness and mucous cell counts in the (-) control and S-group fish during the experiment. The data showed high variability within treatment groups. Figures 1 and 2 show the data trend during the 9-day experiment. It is possible that the temperature manipulations used in this trial did not induce sufficient epidermal damage in sand whiting to facilitate fungal spore infection, thus no EUS lesions or any gross signs of infection were observed during the experiment. Taken all together, a more suitable infection method needed to be developed to further understand the pathogenesis of EUS during low and/or fluctuating temperatures.

Table 1. Sand whiting mortality during the acclimation and experimental period.

Tank no.	Mortality after acclimation	Treatments		Total mortality during experiment
		Temperature	Spore/Stress	
1	0/8	26 °C	spores only	1/8
2	2/8	26 °C	spores only	2/6
3	0/8	26 °C	(-) control	1/8
4	1/8	26 °C	spores+stress	6/7
5	0/8	26 °C	(-) control	0/8
6	1/8	26 °C	spores+stress	6/7
7	1/8	rapid drop	spores only	1/7
8	4/8	rapid drop	spores only	1/4
9	4/8	rapid drop	(-) control	0/4
10	1/8	rapid drop	spores+stress	6/7
11	4/8	rapid drop	(-) control	0/4
12	2/8	rapid drop	spores+stress	4/6
13	3/8	gradual drop	(-) control	1/5
14	1/8	gradual drop	spores only	0/7
15	1/8	gradual drop	spores only	2/7
16	2/8	gradual drop	(-) control	1/6
17	0/8	gradual drop	spores+stress	2/8
18	0/8	gradual drop	spores+stress	3/8

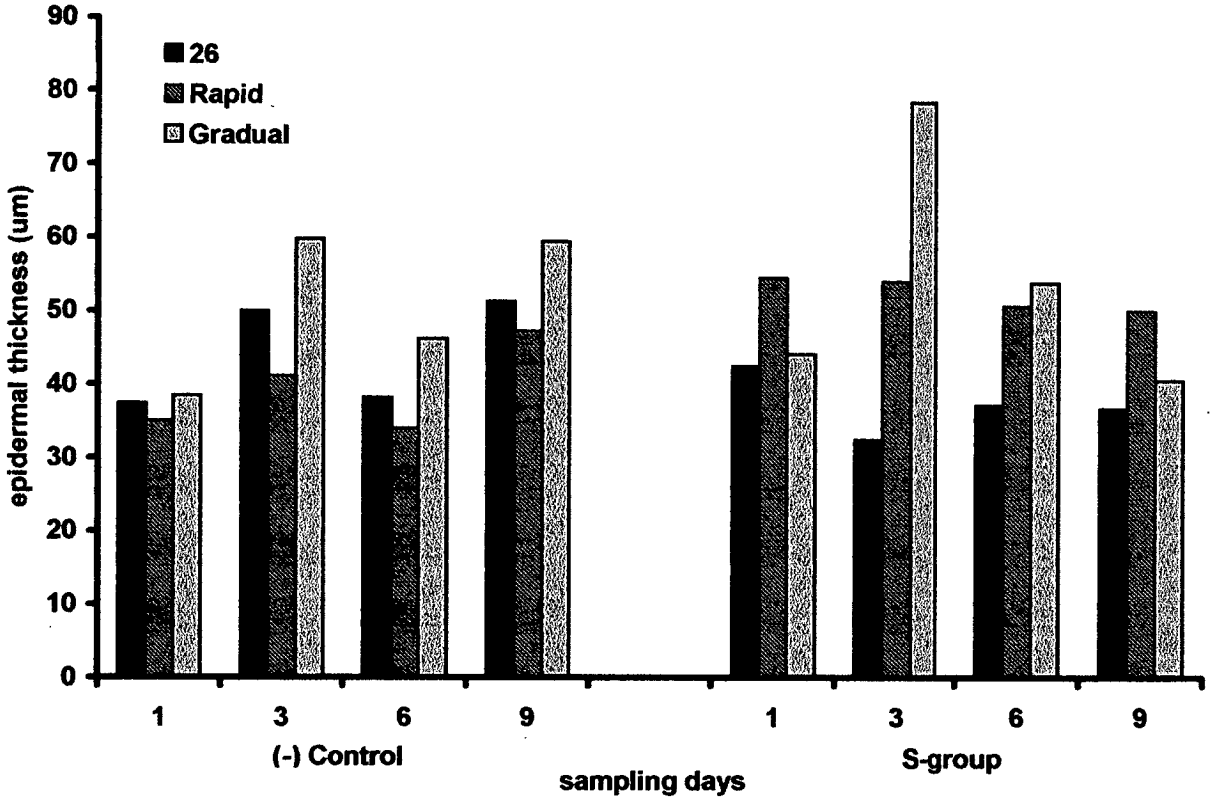

Figure 1. Epidermal thickness measurements in sand whiting subjected to different temperature regimes with or without spore exposure.
Bars are means of 2 replicates of each treatment. ANOVA did not detect any significant difference among treatments ($P<0.05$).

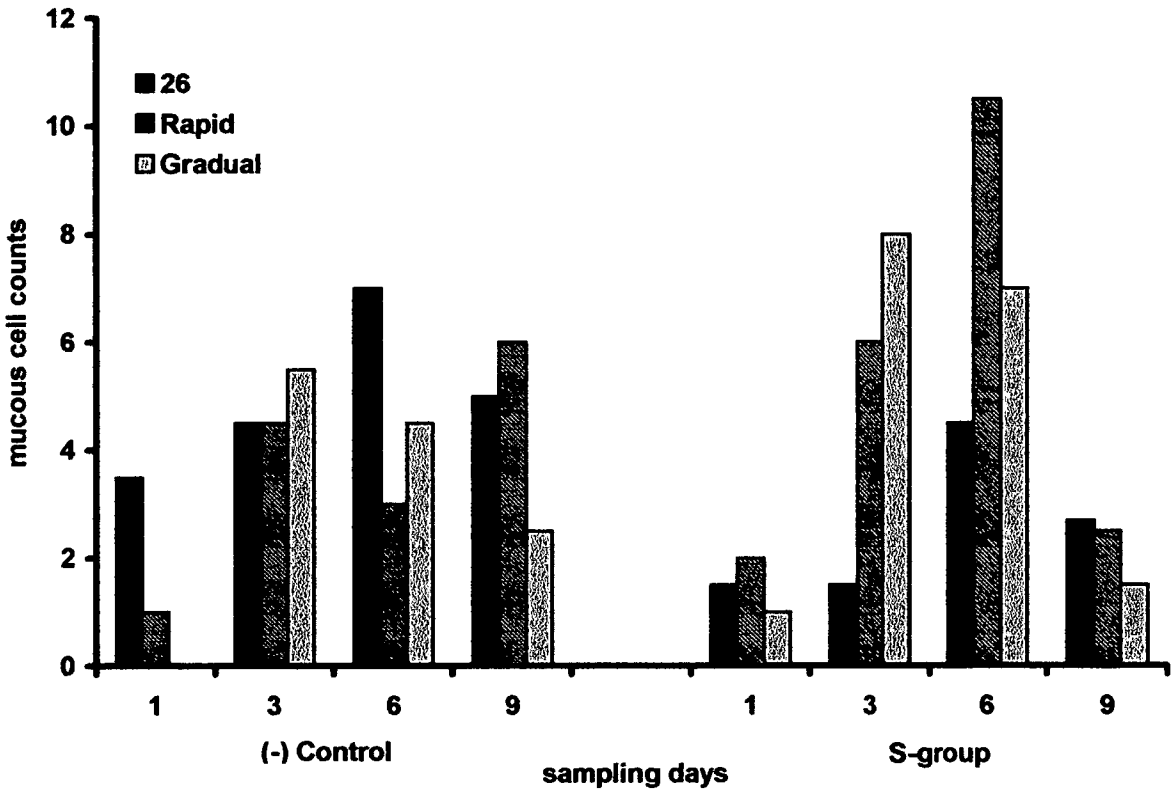

Figure 2. Mucous cells counts in 250 μm of sand whiting epidermis subjected to different temperature regimes with or without spore exposure. Bars are means of 2 replicates of each treatment. ANOVA did not detect any significant difference among treatments ($P<0.05$).

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